Department of Clinical Science, Intervention and Technology,
Division of Ear, Nose and Throat Diseases
Karolinska Institutet, Stockholm, Sweden

LOCAL PHARMACOLOGICAL TREATMENT OF INNER EAR DISORDERS

Cecilia Engmér Berglin

Karolinska Institutet

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"Till örat genom luften med ljudets vågor
fortplantas livets stora frågor.
Men livets alla stora svar,
jag undrar vilken väg de tar?"

Tage Danielsson

CONTENTS

ABSTRACT

Hearing disorders are among the top 10 in terms of burden of disease in middle- and high-income countries, affecting 250 million people worldwide. During the last few decades researchers have made significant advances in understanding the basic mechanisms and molecular biology of inner ear diseases. The principal challenge in treatment of the inner ear is that the targets for pharmacological therapy are inaccessible due to the various barrier systems of the inner ear, and that the inner ear is embedded in the base of the skull. New technologies to provide safe and efficacious delivery of drugs to the inner ear are of great clinical interest. Local administration of medication to the inner ear would solve some of the problems associated with systemic delivery, such as drug interaction and systemic side effects. The aim of the research presented in this thesis was to elucidate different aspects of drug delivery to the inner ear using a local application technique.

The round window membrane (RWM) is believed to be the main route for drug delivery to the inner ear when a drug is administered to the middle ear i.e. by an intratympanic injection. A morphological study of the round window performed on cynomolgus monkey described in Paper I showed the existence of a local defense system housed within the rim of the RWM. Previously undescribed gland-like structures were identified in the loose connective tissue of the mucosal layer near the bony insertion of the RWM. These findings could explain why labyrinthitis is rare despite the close proximity of the infection-prone middle ear. A local immunodefense system would also most likely affect the transport of drugs from the middle ear cavity to the inner ear and needs to be taken into consideration when developing new strategies for local drug administration in the middle ear.

In the studies on which Paper II is based, the rheological and safety aspects of three candidate vehicles for intratympanic drug administration were investigated. The results speak in favor of sodium hyaluronate (HYA gel) which, in contrast to carboxymethyl cellulose and poloxamer 407, did not cause lasting or significant increases in hearing threshold after intratympanic injection in

the guinea pig. Studies of vehicle elimination and morphological investigations support HYA gel as the most promising candidate for intratympanic administration.

An important factor for local administration of drugs to the middle ear aimed for inner ear treatment is the adherence of the vehicle to the RWM. The distribution and elimination of HYA gel after intratympanic injection to the auditory bulla in guinea pig were investigated by magnetic resonance imaging in Paper III. HYA gel was distributed in a predictable way and filled the middle ear cavity well. The HYA gel remained close to the RWM for more than 24 hours. A myringotomy was needed before middle ear administration to allow air to escape and prevent trauma to the RWM.

The hypothesis that higher concentrations of a drug in the inner ear could be achieved by local administration than through systemic administration was investigated in Paper IV and V using the antioxidant thiosulfate, which has previously been identified as a promising otoprotector against cisplatin-induced ototoxicity. The concentration of thiosulfate in scala tympani perilymph was much higher after intratympanic delivery to the guinea pig using an injection of a thiosulfate-containing HYA gel than after i.v. administration of a thiosulfate solution. The levels of thiosulfate in blood remained low after intratympanic administration, confirming that this delivery system will not risk decreased antitumoral effect due to cisplatin inactivation in tumor tissue.

The final study, Paper V, demonstrated that ototoxicity in guinea pigs treated with the antineoplastic drug cisplatin was reduced by injection of thiosulfate-containing HYA gel three hours prior to the systemic cisplatin injection. This confirms the hypothesis of thiosulfate being a promising otoprotector for cisplatin induced hearing loss and also shows that drugs can be delivered locally to the inner ear by intratympanic injection using HYA gel as a vehicle.

LIST OF PUBLICATIONS

This thesis is based on the following original papers, which will be referred to in the text by their Roman numerals.

I. **Engmér C**, Laurell G, Bagger-Sjoback D, Rask-Andersen H. Immunodefence of the round window. *Laryngoscope*. 2008;118(6):1057-62.

II. **Engmér Berglin C***, Videhult Pierre P*, Bramer T, Edsman K, Hultcrantz M, Ekborn A, Laurell G. Local treatment of the inner ear - a study of three different gels aimed for middle ear administration. *In manuscript*.

III. **Engmér Berglin C**, Laurell G, Bramer T, Edsman K, Counter SA, Klason T, Ekborn A. MR imaging of the middle and inner ear following intratympaninic injection of a gadolinium-containing gel. *In manuscript*.

IV. Pierre PV, **Engmér C**, Wallin I, Laurell G, Ehrsson H. High concentrations of thiosulfate in scala tympani perilymph after systemic administration in the guinea pig. *Acta Otolaryngol*. 2009;129(2):132-7.

V. **Engmér C**, Videhult Pierre P, Bramer T, Edsman K, Ehrsson H, Ekborn S, Laurell G. Prevention of cisplatin-induced hearing loss by administration of a thiosulfate containing gel to the middle ear in a guinea pig model. *Cancer Chemother Pharmacol*. 2011; Epub ahead of print.

** Shared first authorship*

ABBREVIATIONS

ABR	auditory brainstem response
BM	basilar membrane
CSF	cerebrospinal fluid
EP	endocochlear potential
Gd-DTPA-BMA	gadolinium-diethylenetriamine pentaacetic acid-bis methylamine
HYA gel	sodium hyaluronan (0.5% w/w)
IHC	inner hair cell
ISSNHL	idiopathic sudden sensorineural hearing loss
MHC	monohydrated cisplatin complex
MRI	magnetic resonance imaging
NaCMC	sodium carboxymethyl cellulose (0.5% w/w)
NaHYA	sodium hyaluronan (0.5% w/w)
OHC	outer hair cell
PLGA	poly lactic co-glycolic acid
POL	poloxamer 407 (25% w/w)
ROS	reactive oxygen species
RWM	round window membrane
SM	scala media
SNHL	sensorineural hearing loss
ST	scala tympani
SV	scala vestibuli
TM	tympanic membrane
WHO	World Health Organization

INTRODUCTION

The present thesis is based on a project with the ultimate goal of developing a model for pharmacological treatment of inner ear disorders by administration of a drug-loaded vehicle to the middle ear cavity.

Ear and hearing

The ear can be divided anatomically into three regions: the external ear, the middle ear and the inner ear. Functionally it is divided into two parts where the external and the middle ear form the conduction part and the inner ear the perception part.

External ear

The external ear consists of the auricle, the external auditory canal and the tympanic membrane (TM) (Figure 1). The fibroelastic cartilage of the auricle is covered with skin. In humans the outer hearing canal is about 3 cm long and S-shaped. The lateral part of the external auditory canal is composed of cartilage covered with skin that contains hair follicles and cerumen-producing sebaceous glands whereas the medial part, located in the temporal bone, is

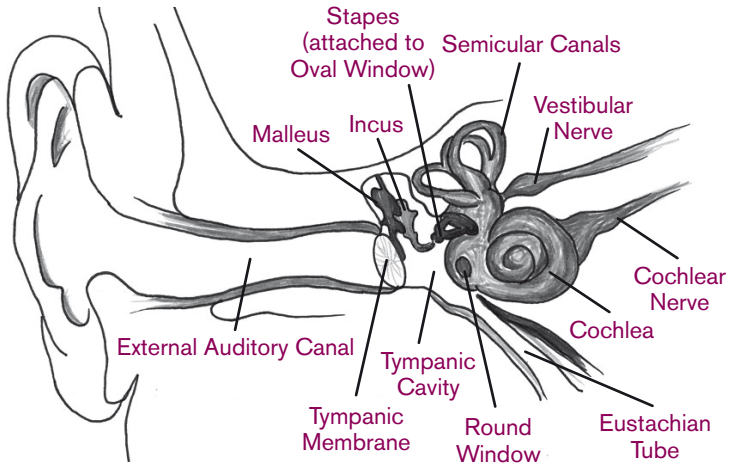

Figure 1. Anatomy of the human ear.

lined by skin without hair follicles and glands.

The primary function of the external ear is to act as a resonator and guide the pressure oscillations of sound to the TM, setting it into vibration.

The middle ear

The middle ear is an air-filled space also called the tympanic cavity (Figure 1). It is limited laterally by the TM and medially by the cochlear eminence. The Eustachian tube (Figure 1) connects the middle ear with the rest of the upper airways. The tympanic cavity harbors the three ossicules named the malleus, the incus and the stapes (Figure 1). They are connected as a chain running from the superior part of the TM, where the long process of the malleus is attached, via the incus to the stapes. The footplate of the stapes is attached to the cochlea at the oval window by the stapedo-vestibular joint. The middle ear epithelium is part of the respiratory mucosa and is lined by a single layer of flat or cuboidal epithelial cells.

Sound waves cause pressure oscillations in the air-filled middle ear, which are transferred to fluid in the inner ear. The different densities of air and liquid, i.e. the different impedances of the media, limit the transfer of sound energy from the air to the inner ear fluid. However, the leverage function of the ossicules and the fact the air interface (TM) is approximately 20 times larger than the fluid interface at the oval window (Figure 1) contribute to impedance matching and thus to the still comparatively efficient transmission of sound energy in the middle ear. This impedance matching is most effective when the pressure in the middle ear is equal to that of the environment. Middle ear pressure is normally adjusted to the varying air pressure of the environment by brief opening of the Eustachian tube during yawning or swallowing.

The inner ear

The inner ear consists of the cochlea (Figure 1) dedicated to hearing, and the vestibular system with its semicircular canals (Figure 1), utricle and saccule dedicated to balance. It is embedded and protected deep within the petrous part of the temporal bone.

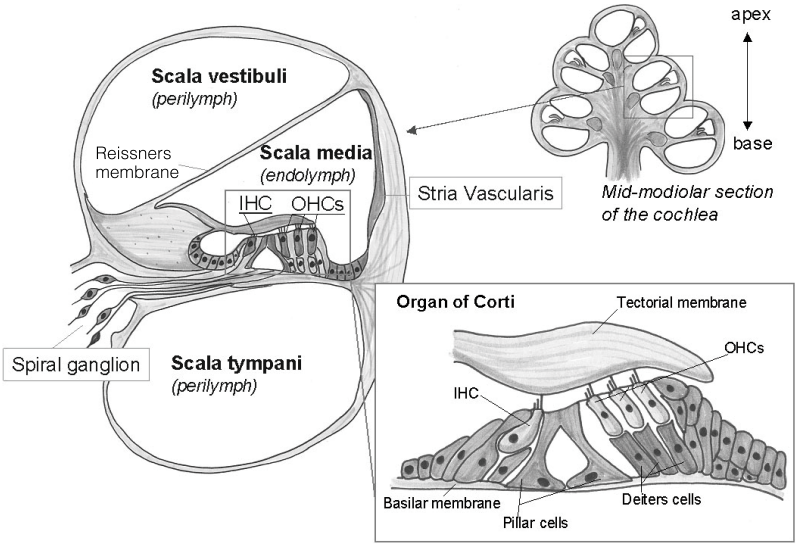

Figure 2. Schematic drawing of the human cochlear anatomy. Top right corner: mid-modiolar section of the cochlea. The left figure illustrates the spiral canal of the cochlea divided into three membranous ducts (scala vestibuli, scala media and scala tympani, which wind around the modiolus to the apex of the cochlea). The Organ of Corti is situated in the scala media containing the inner and outer hair cells (IHC and OHC) (bottom right corner).

The cochlea (Figure 2) has the shape of a coiled shell. Its length varies and the number of turns ranges from two to four depending on species. However the size of the mammalian cochlea is not proportional to the size of the species. The human cochlea is approximately 32 mm long with two and a half turns and in the guinea pig the length is approximately 11 mm with three and a half turns (Wysocki, 2005; Wysocki, 2008). The cochlea consists of three fluid-filled compartments: the scala vestibuli (SV), the scala tympani (ST) and the scala media (SM) (Figure 2). These scalae wind side by side around the central body axis of the cochlea, the modiolus, that contains the spiral ganglion and the cochlear nerve. At the apical end of the cochlea SV and ST merge in the helicotrema. These two scalae are filled with perilymph, a sodium-rich fluid with an ionic composition similar to that of extracellular

fluid whereas SM is filled with endolymph which is comparable to intracellular fluid, rich in potassium and low in sodium (Smith et al., 1954). There is a large positive electro-chemical potential difference between the endolymph and perilymph with the positive endocochlear potential (EP) in the SM maintained by the stria vascularis (Figure 2) in the radial border of SM (Patuzzi, 2011). SM is separated from SV and ST by Reissner's membrane (Figure 2) and the basilar membrane (BM) (Figure 2) respectively. On the BM lies the actual hearing organ, the Organ of Corti, with the inner and outer hair cells (IHCs and OHCs respectively) (Figure 2). The hair cells are organized into one row of IHCs and three rows of OHCs. In humans, each cochlea has about 3000 IHCs and 12000 OHCs (Ulehlova et al., 1987) and in the guinea pig there are about1900 IHCs and 6600 OHCs (Fransson A., personal communication). The cochlear hair cells have stereocilia on their apical surface which project towards and are partly embedded in the tectorial membrane.

From a pharmacological point of view the inner ear can be considered as a three-compartment model, the perilymphatic, the endolymphatic and the sensory tissue compartments.

Besides the oval window, which is covered by the foot-plate of the stapes and is located at the bony enclosure of the SV, the cochlea has another opening called the round window (Figure 1). This window is at ST and is covered by the round window membrane that separates the inner ear from the middle ear.

When acoustic stimulation of the ear reaches the stapes at the oval window, the vibrations cause a pressure difference between SV and ST that will set the BM in movement. The BM is rigid at the base of the cochlea and more flexible at the apical part; this means that a specific frequency will elicit maximum oscillation in a specific region of BM. High frequencies evoke the largest vibration amplitude at the base of the cochlea and low frequencies at the apex. The movement of the basilar membrane creates a shear force relative to the parallel sheet of the tectorial membrane, thus causing deflection of the stereocilia. As a result, ion channels open, the IHCs depolarize and release neurotransmitter to excite

afferent neurons. The nerve impulses generated by the IHCs then travel via the spiral ganglion cells to the auditory nerve and the auditory cortex. The IHCs are regarded as the primary sensory receptors and have a central function in hearing. The OHCs are believed to have dynamic micromechanical properties that contribute to the high sensitivity, high frequency selectivity and wide dynamic range of the hearing organ (Brownell et al., 1985).

Hearing disabilities

Hearing impairment is the most frequent sensory deficit in human populations, affecting more than 250 million people worldwide. According to the World Health Organization (WHO), hearing problems are among the top 10 most common burdens of disease in middle- and high-income countries (Mathers et al., 2000; Mathers and Loncar, 2006). The results of a Swedish study indicate that hearing loss and tinnitus affect more than 30% of the Swedish population (Hasson et al., 2010). Approximately every fifth young adult (under 40 years of age) in Sweden has either tinnitus or hearing loss, indicating that hearing problems may be even more common in the future (Hasson et al., 2010). The prevalence of hearing loss and tinnitus is age-related but also influenced by cumulative exposure to noise (Henry et al., 2005; Nelson et al., 2005). Men are more often affected than women (Agrawal et al., 2008).

Hearing losses can be divided into two main types, conductive and sensorineural. Conductive hearing loss is caused by abnormalities or obstruction of the outer and middle ear that hamper the sound on its way to the cochlea. Among the various causes of conductive hearing loss are inflammation, or congenital malformation, of the outer or middle ear. Conductive hearing loss can often be treated surgically, medically or with hearing aids. Sensorineural hearing loss (SNHL) is classified either as a cochlear hearing loss, when the hearing loss is caused by abnormalities of the cochlea, or as a retrocochlear hearing loss, when the abnormalities are located in the auditory nerve or the auditory centers at the brainstem or the cortex of the brain. The

cochlea is the most frequently affected site among people with hearing disabilities of the sensorineural type. The causes of SNHL include heritable diseases, noise exposure, autoimmune disease, meningitis, head trauma, tumors, and ototoxic lesions; age-related factors also play a role. A person with SNHL may not only have a reduced ability to hear faint sounds but often also problems with tinnitus, difficulties interpreting speech in noisy surroundings, and hyperacusis. Humans and other mammals have no ability to restore damaged inner and outer hair cells or neurons and there is to date no cure for SNHL. Functional improvement can be achieved with hearing aids that work as amplification devices and with cochlear implants that stimulate auditory neurons electrically. New drugs and innovative drug delivery systems are being developed to treat various inner ear ailments such as ototoxicity, autoimmune inner ear disease, idiopathic sudden sensorineural hearing loss (ISSNHL) and for regenerating sensory cells and preserving neurons. Progress in the field of inner ear drug delivery would ultimately lead to improved quality of life for the large group of patients suffering from hearing-related disorders.

Systemic treatment of the inner ear

Systemic administration of medications directed to the inner ear consists of dosing medication via the oral, intravenous or intramuscular route. Streptomycin was one of the first medications to be used for systemically treatment of inner ear disease. This drug had been found to be ototoxic soon after its discovery and as early as 1948 it was recognized that the ototoxic effect could be put to use as treatment to control vertigo (Fowler, 1948). The most common indication for streptomycin today in inner ear treatment is severe bilateral Ménière's disease, for which the drug is given intramuscularly and titrated according to clinical effect (Berryhill and Graham, 2002; Sataloff et al., 1996). Another example of systemic treatment of inner ear disorder is systemic steroids, which have become a standard treatment worldwide for ISSNHL. However, the efficacy of this treatment has been questioned on the basis of methodological deficiencies and lack of well-designed

randomized controlled clinical trials with adequate power (Conlin and Parnes, 2007; Wei et al., 2006). Other drugs that have been delivered systemically to target the inner ear are diuretics for Ménière's disease (Coelho and Lalwani, 2008), N-acetylcysteine for noise-induced hearing loss (Coleman et al., 2007; Kopke et al., 2007) and bisphosphonates for otosclerosis (Brookler, 2008). The literature contains very few prospective, randomized studies on pharmacological treatment of well-defined inner ear disorders. Most studies are based on a small number of patients given a specific drug, and lack a control group.

However, clinical use of systemically administered medications for treatment of inner ear disorders raises many concerns, such as unacceptable side-effects and drug interactions in the blood compartment (Ekborn et al., 2002). Moreover, it may be difficult for a drug to reach the inner ear from the systemic circulation due to various barrier systems between the blood compartment and inner ear compartments (Juhn, 1988; Sterkers et al., 1982). These disadvantages make local drug delivery attractive.

Local treatment of the inner ear

In the search for safe and effective medical treatment of the inner ear, various techniques have been developed that permit local drug delivery either to the middle ear or directly to the inner ear.

Intratympanic injection

Injection to the middle ear with a needle, through a myringotomy or through a tympanostomy tube is the simplest way to deliver medication aimed for inner ear treatment. This technique uses the middle ear as a reservoir for drug transport to deeper compartments. It is minimally invasive, quick and easy to repeat if necessary. Intratympanic injection can be performed in an outpatient setting and the technique is currently in widespread use. The level of a drug in perilymph after application to the middle ear is influenced by the drug's ability to permeate through the round window membrane (RWM) and by its residence time, which is determined by how fast the drug is eliminated from the round window niche

by the middle ear mucosa and how fast the injected solution is drained from the middle ear through the Eustachian tube. These factors might explain the large variability in the results reported from pre-clinical and clinical trials on the effect of intratympanic injections. Development of a method that delivers a fixed dose and ensures contact with the RWM would be of great importance for solving the problems relating to drug delivery to the inner ear by intratympanic administration.

The first local drug delivery to the inner ear of humans was developed in the 1950s for treatment of Ménière's disease with local anesthetics (Ersner et al., 1951) and antibiotics (Schuknecht, 1956). Today intratympanic application of gentamicin is a common strategy for the treatment of Ménière's disease (Ghossaini and Wazen, 2006). Another method currently in widespread use to treat inner ear disease is intratympanic steroid therapy, which has been shown to be effective against Ménière's disease in some studies (Boleas-Aguirre et al., 2008; Garduno-Anaya et al., 2005), but had yielded discouraging results in others (Silverstein et al., 1998). However, intratympanic delivery of steroids is best known for the treatment of ISSNHL in which context it has been used as salvage therapy after systemic treatment with steroids has failed (Lefebvre and Staecker, 2002; Slattery et al., 2005) or as combination therapy when a high dose of prednisone is being tapered off (Battaglia et al., 2008; Lautermann et al., 2005). However, none of these studies drew any definitive conclusions based on statistically significant differences between study groups, and the trials were not randomized. Some studies showed good outcome of locally administered prednisone and some did not. In order to resolve some of the controversy concerning therapy for ISSNHL the National Institute of Health sponsored a recently published multicenter treatment trial which concluded that intratympanic steroid treatment was not inferior to oral steroid treatment and could therefore be a suitable alternative if there are medical contraindications to oral steroids (Rauch et al., 2011). Other disorders that have been treated with intratympanic steroids are

autoimmune inner ear disease (Light and Silverstein, 2004) and tinnitus (Dodson and Sismanis, 2004).

Local application by intratympanic injection has been used experimentally for a variety of therapeutic agents, including antibiotics, steroids, anti-oxidants and neurotrophins. Efforts to control the variability in drug delivery to the inner ear have led to the development of volume stabilizing vehicles aimed for middle ear administration. These vehicles consist of biodegradable polymers or hydrogel delivery systems into which the drug can be loaded. The release of the drug is then dependent on the degradation of the material and drug diffusion from the vehicle. Examples of biodegradable vehicles that have been used for middle ear application are natural materials like hyaluronan (Borden et al., 2011; Kelly et al., 1999; Saber et al., 2009), gelatin (Endo et al., 2005; Ito et al., 2005) and chitosan (Paulson et al., 2008; Saber et al., 2010; Xu et al., 2010) and synthetic polymers such as poly lactic co-glycolic acid (Horie et al., 2010; Tamura et al., 2005) and poloxamers (Piu et al., 2011; Salt et al., 2010; Wang et al., 2009). Of these, only hyaluronan and poloxamers have been used as vehicles for application in which the middle ear has been filled. All the other materials have been used for middle ear administration as described below. One major drawback to the use of intratympanic injection is the transient conductive hearing loss that the patient experiences when the middle ear is filled with the vehicle. Another is the potential reaction of the middle ear mucosa to the drug or vehicle.

Round window administration

As the RWM is believed to be the most important interface for drug uptake to the inner ear after middle ear administration, various techniques for drug application to the round window have been developed. These techniques are described below and the properties of the round window membrane will be discussed in the section about drug transport to the inner ear.

The delivery system called the MicroWick is a small wick (1 x 9 mm) composed of polyvinyl acetate that can be inserted

through a tympanic membrane ventilation tube into the round window niche (Silverstein, 1999). The patient can then self-administer eardrops into the ear canal, where they are absorbed by the wick and transported to the RWM. The MicroWick has successfully been used in clinical studies to deliver gentamicin in order to treat Ménière's disease (Hill et al., 2006; Suryanarayanan et al., 2009) and to deliver methylprednisolon for ISSNHL (Van Wijck et al., 2007). Potential complications of treatment with MicroWick are infections of the middle or external ear, tissue ingrowth in the middle ear leading to fibrosis or cholesteatoma and the development of a persistent perforation of the tympanic membrane (Robey et al., 2010).

The implantable microcatheter method can be used to deliver medication continuously to the RWM. This technique involves implanting a microcatheter via a well in the posterior part of the external auditory canal and through a tunnel in the eardrum to the round window niche. The system makes it possible to deliver medication continuously for several weeks. It has been used to deliver steroids to the RWM in patients with ISSNHL (Herr and Marzo, 2005; Kopke et al., 2001; Plontke et al., 2009; Sun et al., 2007) and gentamicin in patients with Ménière's disease (Charabi et al., 2000; Hoffer et al., 2001; Suryanarayanan and Cook, 2004). However, this delivery system involves surgical intervention with its associated risks, as well as the risk of persistent perforation of the tympanic membrane and the risk that the patient will accidentally dislocate the catheter (Plontke et al., 2006).

As mentioned above, biodegradable vehicles including gelatin, chitosan and poly lactic co-glycolic acid have been used in experimental studies for drug application to the RWM. Among the drugs that have been delivered to the inner ear by this method are brain-derived neurotrophic factor (Endo et al., 2005; Havenith et al., 2011), insulin-like growth factor (Fujiwara et al., 2008; Lee et al., 2007) and dexamethasone (Paulson et al., 2008). This drug delivery system ensures that the drug is in close contact with the RWM and the medication can be released, e.g. by hydrolysis in a controlled fashion or by diffusion out of the matrix. The vehicle

used can also be altered to change the dynamics of drug release (Paulson et al., 2008). The main disadvantage of this method is that surgical intervention is required for proper placement in the round window niche. Compared to the middle ear injection, where the entire middle ear is filled, the risk of transient conductive hearing loss is smaller, but on the other hand, since the volume applied at the round window is small, it might not contain enough medication for the desired treatment time.

Nanotechnology is an expanding scientific field and is expected to have far-reaching effects in many fields of medicine. Nano-otology denotes nanomedicine as applied to diseases and disorders of the ear. By definition, a nanoparticle has a diameter of less than 1000 nm; those used for drug delivery to the inner ear are often 200 nm or less (Hornyak, 2005). Nanoparticles have been shown to reach the cochlea when administered systemically but the intracochlear concentration is higher when the particles are applied to the RWM (Tamura et al., 2005). To date, however, there is no published evidence of successful treatment of inner ear disorders using nanoparticles as drug carriers.

Intracochlear drug delivery

Drugs delivered directly into the cochlea have higher bioavailability in the cochlear tissue than drugs applied topically in the middle ear, and also have more direct access to sensory cells and neurons. Intracochlear drug delivery is performed either by a cochleostomy through the otic capsule (typically to the ST but both SM and SV have been explored as routes for delivery) or by injection through the RWM. Technical solutions designed to deliver drugs directly to the inner ear consist of micro-pumps with active or passive control systems, alone or in combination with a cochlear implant.

Osmotic pumps were the first intracochlear delivery system to be explored (Kingma et al., 1992). They operate by establishing an osmotic gradient that drives the drug out of a canister – and in the case of intracochlear delivery through a cannula into the cochlea – at a rate determined by the design of the device (Borenstein, 2011). In experimental studies for example brain-derived neurotrophic

factor (Agterberg et al., 2009; McGuinness and Shepherd, 2005), dexamethasone (Takemura et al., 2004), gentamicin (Lii et al., 2004) and thiourea (Ekborn et al., 2003) have been delivered using osmotic pumps. Among the disadvantages of this method are that investigators cannot stop and restart the treatment without removing the system, cannot vary dosage and dosing intervals, and cannot refill the pump.

Direct injection to the cochlea has been used for gene transfer and delivery of liposomes and agents capable of reducing damage associated with cochlear implant (Oestreicher et al., 1999; Praetorius et al., 2007; Stover et al., 1999). Another delivery system is the syringe pump delivery that has been used experimentally to establish kinetic models for drug transport in the ST (Borkholder et al., 2010; Plontke et al., 2007). These two constant infusion approaches are, however, hindered by the low rate of clearance of cochlear fluids and thus the limited volume of a drug that can be administered during a certain time without causing injury to the cochlea. The cochlear scalae are relatively long and narrow passages and the apical part of the cochlea is difficult to reach with surgery. Since the cochleostomy is often made in the basal part of the cochlea, it is difficult getting drugs to the apical region. A reciprocating distribution system for intracochlear delivery has therefore been developed in which a volume of drug is infused and the same amount of perilymph is withdrawn in a cyclic manner without any accompanying change in fluid volume within the cochlea (Chen et al., 2005; Fiering et al., 2009; Pararas et al., 2011; Sewell et al., 2009). A recirculation component of the delivery device permits delivery of a drug at a constant concentration over an extended period of time – potentially for years – without any need to refill the reservoir. So far a few studies on laboratory animals have been performed using this new delivery system in order to examine drug kinetics within the cochlea. The drawbacks of this delivery system are associated with the surgical intervention needed for implantation.

A cochlear implant is a device that provides a sense of sound to a person who is profoundly deaf or severely hard of hearing

by direct stimulation of the auditory nerve. It comprises a microphone, speech processor, transmitter, receiver and electrode array. The latter is implanted into the inner ear via a cochleostomy or through the RWM, which makes the cochlear implant a pathway for drug delivery to the inner ear. Delivery systems that have been integrated with a cochlear implant include the use of biorelease polymers coated onto the implant electrodes (Dinh et al., 2008; Hendricks et al., 2008; Richardson et al., 2009) and the connection of external pump systems (Paasche et al., 2006; Shepherd and Xu, 2002). In experimental studies, neurotrophic factors aiming to preserve spiral ganglion cells (Richardson et al., 2009) and dexamethasone (Dinh et al., 2008) have been delivered using this route. Side effects of modified cochlear implants for drug distribution to the inner ear could be greater risk of infection and disturbed function of the implant.

Drug transport to the inner ear

Systemic administration

Drugs administered systemically have to pass the blood-cochlear barrier to reach the inner ear compartments. The blood-cochlear barrier is physiologically similar to the blood-brain barrier (Juhn and Rybak, 1981; Juhn, 1988). It consists of a continuous capillary endothelium lining blood vessels in the cochlea and these endothelial cells are connected with tight junctions (Jahnke, 1975; Kimura and Ota, 1974). This means that drugs in the blood compartment have to be actively or passively transported across the capillary endothelia. Drug molecules that are large or highly charged will have greater difficulty crossing the barrier than small drugs with high lipid solubility. High capacity to bind to protein will also increase a drug's ability to cross. The positive potential of SM is another obstacle to drug entry and positively charged drugs tend not to enter SM since the electrical gradient works against them (Salt et al., 1991b; Salt and DeMott, 1995). Components such as enzymes and cellular uptake systems within the blood-cochlear barrier might also affect the concentration of medication

that reaches the cochlea from the blood compartment, but these are not yet well characterized (Swan et al., 2008).

Local administration to the middle ear

There is experimental support that drugs can be taken up into the inner ear over several interfaces between the middle and the inner ear including the RWM, the oval window and the bony otic capsule.

The round window membrane

The RWM is localized in the medial wall of the middle ear within the round window niche at the basal end of the cochlea. In the human being, the RWM is approximately 70 µm thick (Carpenter et al., 1989) and in the guinea pig about 12 µm. It consists of three layers: an outer epithelium facing the middle ear cavity, a core of connective tissue and an inner epithelium facing the ST of the inner ear (Goycoolea and Lundman, 1997). The outer epithelium consists of a single layer of low cuboidal to flat cells on a basal membrane and is a continuation of the middle ear mucosa. Tight junctions connect the cells of the outer epithelium and the core contains connective tissue fibroblasts, fibrocytes, collagen, elastin, capillaries, and nerves (Schachern et al., 1982). The inner epithelial layer is a continuation of the cell lining in the ST and the cells are flat, squamous and overlap each other (Goycoolea, 2001).

The basic function of the RWM is believed to be to permit displacements and changes of fluid pressure in the inner ear when the stapes footplate in the oval window is put in motion by sound stimulation (Goycoolea and Lundman, 1997; Wever and Lawrence, 1948). The RWM has also been suggested as an alternative route for sound stimulation (Goycoolea and Lundman, 1997). Even though the middle ear is an area prone to infections, the spread of microorganisms to the inner ear is very rare. The RWM most probably takes part in a local defense system, undergoing histopathological changes when exposed to injury (Goycoolea and Lundman, 1997; Goycoolea, 2001). For passage of substances from the middle to the inner ear, the RWM is believed to be the most important route and may act as a semi-permeable membrane

(Goycoolea, 2001; Lundman, 1993). The thickness of the RWM affects its permeability (Goycoolea et al., 1988b). The severity and the duration of a middle ear inflammation affect both the thickness and the permeability of the RWM. Its permeability is increased in the early phase of otitis media, but the membrane becomes thicker and more impermeable at later stages of disease (Ikeda and Morizono, 1988; Kim et al., 1990; Lundman et al., 1992; Schachern et al., 1987). A substance's passage over the RWM is dependent on its size, structure and electrical charge. Substances with low molecular weight (<1 kDa) such as sodium ions, gentamicin, neomycin and streptomycin pass readily through the RWM (Juhn et al., 1989). Substances with high molecular weight (>10 kDa) such as ferritin, albumin and endotoxins, on the other hand, can only be transported through the membrane by pinocytocis (Juhn et al., 1989). The positively charged ferritin ion can be transported across the membrane (Goycoolea et al., 1988a; Goycoolea et al., 1988b) whereas the negatively charged ferritin ion cannot (Goycoolea et al., 1988b). A number of substances can increase the permeability of the RWM such as histamine, leukotrienes and prostaglandins (Juhn et al., 1989) as well as exotoxin of Staphylococcus aureus and endotoxin of Escherichia coli (Engel et al., 1995; Engel et al., 1998; Ikeda and Morizono, 1988). Drug solution osmolarity, benzyl alcohol content, and possible injury to the RWM during suctioning of the middle ear are also factors that influence the permeability (Mikulec et al., 2008).

The oval window

The oval window consists of the stapes footplate and the annular ligament. This structure has been suggested as a secondary route of passage for particles from the middle ear cavity to the inner ear (Saijo and Kimura, 1984; Tanaka and Motomura, 1981). However, it is technically difficult to measure the amount entering the inner ear through this route. Attempts to occlude the RWM e.g. with dental cement (Saijo and Kimura, 1984) have not been fully successful. There is also a radial communication between the perilymphatic scalae of the cochlea (Salt et al., 1991a; Salt et al., 1991b), making it even more difficult to draw any conclusion

about drug entry through the oval window based only drug concentrations in the SV or the saccule. Thus the possibility of any drug uptake through the oval window is still uncertain but if it occurs, it is most probably influenced by drug size and charge. Even under optimal conditions, the amount of drug that can be taken up via this pathway is thought to be small compared to that entering though the RWM (Salt and Plontke, 2009).

The bony otic capsule

It has recently been shown that drugs can enter the perilymph through the thin bony otic capsule in the apical turns of the guinea pig cochlea when the drug is applied by intratympanic injection and the middle ear is filled with solution (Mikulec et al., 2009). In humans, the bone of the otic capsule is thicker than in the guinea pig and drugs will probably not penetrate to the same extent. This illustrates a considerable problem of extrapolating results from animal studies to human beings since drug distribution patterns along the length of the cochlea are likely to differ.

Drug distribution within the inner ear

Knowledge on drug distribution, or drug trafficking, within the inner ear is rather limited. Many of the structures in the inner ear are in diffusional continuity with the perilymph. A drug can therefore diffuse from the perilymph into the Organ of Corti, with its hair cells, neural terminals and other specialized supporting cells (Ilberg and Vosteen, 1969). Drugs might also be able to enter the modiolus and thus reach the spiral ganglion through numerous canaliculi on the surface of the osseous spiral lamina (Rask-Andersen et al., 2006; Schuknecht and Seifi, 1963). SV and ST are in direct communication via the helicotrema at the apex of the cochlea. The rate of longitudinal flow of perilymph in the cochlea is reported to be very slow, less than 2 nl/min (Ohyama et al., 1988), and drug distribution via this route is therefore limited to passive diffusion. Because of the relatively long distance between the basal turn of ST and the basal turn of SV transport via the helicotrema would be extremely slow. The possibility of a

radial communication has been suggested (Duvall, 1972; Konishi et al., 1978; Saijo and Kimura, 1984; Salt et al., 1991a; Salt et al., 1991b) and is presumed to occur through the loose and fibrous intracellular spaces of the spiral ligament.

An intracochlear barrier, the perilymph-endolymph barrier, restricts entry of drugs and other substances from the perilymphatic compartment to the endolymphatic compartment (Laurell et al., 1995b; Salt et al., 1991b; Salt and DeMott, 1995). The cells lining the endolymphatic space including the stria vascularis are connected through tight junctions (Jahnke, 1975) and the perilymph-endolymph barrier is semi-permeable or impermeable for substances other than water (Hirt et al., 2010; Sterkers et al., 1982; Sterkers et al., 1987). The entry of substances to the endolymph via the perilymphatic space is likely to depend on the electrical charge due to the endocochlear potential. Cationic markers have been shown to be excluded from endolymph (Salt et al., 1991b) whereas anionic markers are accumulated and retained (Salt and DeMott, 1995).

How drugs reach and are taken up by the inner and outer hair cells is not fully understood. Besides the perilymphatic route, an endolymphatic pathway and uptake of drugs through the apical parts of the inner and outer hair cells has been suggested (Steyger et al., 2003; Wang and Steyger, 2009).

Metabolism and elimination from the inner ear

Little is known about how specific groups of drugs are metabolized and eliminated after uptake to the inner ear. The perilymph probably harbors enzymes similar to those in plasma, that can alter the structure of drugs. Barriers such as tight junctions that limit drug uptake to the inner ear will most likely also limit elimination of drugs once they have entered the inner ear compartments, leading to slow elimination (Hellberg et al., 2010). The major capillary beds within the inner ear are associated with the spiral ganglion, the stria vascularis and the spiral limbus. Communications between the ST and the spiral ganglion (Shepherd and Colreavy, 2004) and between the SV and the modiolar space (Rask-Andersen et

al., 2006) have been demonstrated and elimination of drugs from the perilymphatic compartment probably occurs through the vascularized tissues that lie closest to the perilymph. The rate of elimination from the perilymphatic compartment is an important factor for drug concentration in the cochlea, which in turn affects the distribution of the drug towards the apex following, e.g., drug application to the RWM. A steady state will be achieved at a specific region in the perilymphatic space when the amount of a drug eliminated is equal to the diffusion rate along the scala at the same point. The drug concentration at more apical regions will therefore not exceed the concentration at the base of the cochlea.

Cisplatin-induced ototoxicity

In this thesis cisplatin has been used as a model for exogenous inner ear injury. Moreover, cisplatin-induced ototoxicity is a major clinical problem and improved knowledge about prevention of cisplatin ototoxicity would be of great importance.

Cisplatin (Figure 3) is a chemotherapeutic agent that has been widely used for more than three decades against various solid malignant tumors. Its dose-limiting side effects are nephrotoxicity and ototoxicity (Rabik and Dolan, 2007). While nephrotoxicity can be prevented by increased hydration and forced diuresis, there is still no way to prevent cisplatin's ototoxicity or repair the damage it causes. In pediatric cancer patients, loss of hearing is particularly disabling as it may hamper the child's speech,

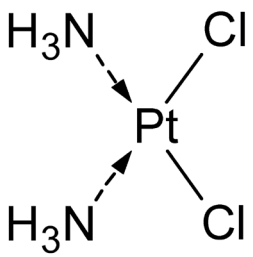

Figure 3. The molecular formula of the chemotherapeutic agent cisplatin.

cognitive, and social development. Cisplatin-induced hearing loss may lead to discontinuation of cisplatin therapy (de Jongh et al., 2003; Ekborn et al., 2004). The cisplatin analog carboplatin is less ototoxic but although a switch to carboplatin treatment reduces the risk of hearing loss, it can also reduce the chance of cure or the time to progression of the cancer (Go and Adjei, 1999).

Hearing loss has been a known side effect ever since cisplatin was introduced clinically in the beginning of the 1970s (Higby et al., 1974; Rossof et al., 1972). The first signs of ototoxicity include hearing loss in the high frequency range, which rarely affects hearing perception (Blakley et al., 1994; Coupland et al., 1991; Higby et al., 1974; Laurell and Borg, 1988; Schaefer et al., 1985), along with transient or permanent tinnitus (de Jongh et al., 2003; Ekborn et al., 2004; Higby et al., 1974; Laurell et al., 1996). The hearing loss normally develops over days (Grau et al., 1996). Patients receiving high dose treatment may develop severe hearing loss at lower frequencies, including the speech frequency range (Blakley et al., 1994; Coupland et al., 1991; Ekborn et al., 2004; Schaefer et al., 1985). Risk factors for cisplatin-induced hearing loss include treatment with a single high dose (Laurell and Jungnelius, 1990) or a high cumulative dose (Coupland et al., 1991; Laurell and Jungnelius, 1990), concomitant radiation towards the base of the skull (Kolinsky et al., 2010) and young age in pediatric patients (Coupland et al., 1991). There is great inter-individual variability in ototoxicity; the reason for this is not known but it might be explained by differences in metabolic status, pharmacokinetics and genetic factors. In a study of survivors of testicular cancer, cisplatin-induced long-term hearing impairment was found to be associated with specific genotypes of glutathione S-transferase (Oldenburg et al., 2007), an enzyme known to be involved in the glutathione antioxidant system.

The primary cytotoxic effects of cisplatin are believed to be mediated by the monohydrated cisplatin complex (MHC), which reacts with nuclear DNA, forming platinum-DNA adducts (Siddik, 2003). The cytotoxicity includes the generation of reactive oxygen species (ROS) (Kim et al., 2010). The main targets of the

ototoxic effects seem to be the OHC in the Organ of Corti, the spiral ganglion cells, and the cells of the stria vascularis in the basal part of the cochlea (Rybak, 2007). One explanation for the differences in toxicity along the length of the cochlea could be that the OHCs in the basal part contain significantly lower levels of the antioxidant glutathione than those in the apical part (Sha et al., 2001). Another explanation could that the concentration of cisplatin is higher at the base of the cochlea than at the apex (Hellberg et al., 2010).

Since one of the mechanisms behind cisplatin-induced ototoxicity is believed to be the generation of oxidative stress, antioxidants have long been in focus in the search for otoprotection (Choe et al., 2004; Nader et al., 2010; Wang et al., 2003; Wimmer et al., 2004). Several sulfur-containing compounds are antioxidants with high affinity to platinum species (Videhult et al., 2006). They may prevent cisplatin- and MHC-induced toxicity in a dual manner, both by decreasing the effect of ROS and by chelating cisplatin and MHC, forming inactive complexes. Among the sulfur-containing antioxidants that have shown otoprotective potential in experimental studies are N-acetyl-cysteine (Dickey et al., 2005), sodium thiosulfate (Dickey et al., 2005), and D-methionine (Campbell et al., 1996). However, when administered systemically, such compounds may reduce the efficacy of cisplatin-based chemotherapy due to drug interactions (Ekborn et al., 2002). Moreover, the uptake of antioxidants from the systemic circulation to the cochlea is limited due to the blood-labyrinth barrier. Intracochlear administration of otoprotective compounds is not a clinical option since the human cochlea is located in the base of the skull and such treatment would involve invasive surgery that may cause irreversible injury to aural sensory structures. Because of these limitations there is growing interest in techniques that deliver otoprotective substances to the inner ear by way of local administration to the middle ear. The fact that it might be possible to achieve higher concentrations of a drug by local delivery compared to systemic delivery also speak in favor of the

local administration systems. For clinical application, effective, safe and direct methods for delivery of therapeutic molecules to the inner ear need to be developed.

AIMS

The overall aim of the research project presented in this thesis was to develop a method for local administration of drugs to the middle ear by using a suitable vehicle that can be loaded with different kinds of drugs or a combination of drugs for protection of the inner ear or treatment of inner ear disease. The specific aims of each individual paper were:

I. To better describe the morphological and immunological properties of the round window membrane.
II. To determine the safety of intratympanic administration of vehicle to the middle ear by studying the effect of three different gels on the middle and inner ear as well as the elimination of the gels from the middle ear.
III. To investigate the distribution and elimination of a gadolinium-containing gel after injection to the middle ear *in vivo* using magnetic resonance tomography.
IV. To study the uptake of a presumed otoprotector to the inner ear after systemic administration *in vivo*.
V. To study the uptake of the same presumed otoprotector to the inner ear after local administration of a vehicle to the middle ear and to study the potential effect of the otoprotector against experimental inner ear damage *in vivo*.

MATERIALS AND METHODS

Laboratory animals (Papers I-V)

All experimental animal studies were performed with permission of the local ethical committees for experiments on laboratory animals, Direction Départementale des Services Vétérinaires du Rhône (France) for the studies performed on cynomolgus monkeys and Stockholms Norra Djurförsöksetiska nämnd (Sweden) for the studies performed on guinea pigs.

Cynomolgus monkeys (Paper I)

The monkey is closely related to man, having close genetic and anatomical similarities. The ears used in Paper I originated from cynomolgus monkeys that had been used in a pilot study of a commercial drug preceding a phase I study on patients. The non-injected and the saline-injected control ears, a total of 8 ears from 6 monkeys, were a kind gift from the pharmaceutical company.
Ethical permission number: C69-489 and B69-489

Guinea pigs (Papers II-V)

The guinea pig is commonly used in hearing research. It is easily bred in captivity. Because the guinea pig cochlea protrudes into the middle ear and is not encapsulated in the temporal bone as in humans and many other species, the inner ear of the guinea pig is accessible for physiological, pharmacological and histological studies. The behavioral hearing thresholds are similar to those of humans (Prosen et al., 1978). In paper II-V 88 albino guinea pigs of both sexes from a local breeder were used.
Ethical permission number: N334/05, N50/07 and N334/08

Auditory brain stem responses (Papers I, II and V)

To analyze electrophysiological hearing thresholds the animals auditory brainstem response (ABR) was tested. This is a widely used method in which an electric response from nerve cells in the auditory pathway is elicited by brief auditory stimuli and

recorded via electrodes mounted subcutaneously on the scalp. A characteristic curve is obtained with vertex positive waves, numbered I-V, corresponding to the different stations in the auditory pathway from the cochlea to the brainstem. The electrophysiological hearing thresholds of the animals were determined by varying the intensity of the stimulus in 5 dB steps around the electrophysiological hearing threshold and observing the resulting curves. The electrophysiological hearing threshold was defined as the lowest level at which a reproducible response could be recorded. In Paper I ABR patterns were recorded using equipment from Interacoustics AS, model EP 25 (Assens, Denmark) and in Paper II and V measurements were performed with a TDT system II (Tucker Davies Technologies, Gainesville, FL, USA). The sound stimuli were delivered inside the ear canal through a speculum from a high frequency transducer with the animal placed in a soundproof test box. In Paper II one group of animals was used to evaluate whether the vehicles had deleterious effects on hearing as assessed using acoustically evoked ABR assessments. Thresholds were determined before injection of gel to the middle ear cavity and then ABR assessments were repeated weekly for three weeks after the administration. In Paper I and in the otoprotection study of Paper V the electrophysiological hearing thresholds of the animals were verified as being normal before start of the experiment and treatment.

Morphological examination using light (Papers I and II) and electron microscopy (Paper (I)

The anesthetized monkeys in Paper I were fixed through intracardiac perfusion and the ears were dissected out. After decalcification, the temporal bones were stained in osmium tetroxide. The round window niche was dissected out, positioned, and sectioned serially at a transverse cutting angle and subsequently embedded in EPON epoxy resin and sectioned for light microscopy and transmission electron microscopy. Serial semi-thin sections for light microscopy were made and every 10th section stained with toluidine blue. The specimens were observed and

photographed in a Zeiss Axiophoto microscope (Jena, Germany). Areas of interest were sectioned for transmission electron microscopy through a re-embedding technique. The semi-thin sections were glued on a plastic holder, and ultra-thin sections were cut and stained with lead citrate and uranyl acetate and then examined and photographed in a transmission electron microscope, JEOL 100 SX TEM (Tokyo, Japan).

The auditory bullae of the guinea pigs that were to be used for light microscopic examination in Paper II were quickly disassembled from the temporal bone and fixed after decapitation of the deeply anesthetized animals. The bullae were decalcified, embedded in JB4 resin (Polyscience), and sectioned at 3-µm thickness with a rotary microtome (Microm HM 355 S). The left ears were sectioned perpendicularly to the RMW and across the cochlea. The right ears were sectioned perpendicularly both to the RWM and the TM across the middle ear and the cochlea. Every 10th section was mounted on a glass slide, stained with toluidine blue, and examined under a light microscope (Zeiss) equipped with a digital camera (Altra 20 soft imaging system, Olympus). The thickness of the RWM and the TM was measured using image analysis software (Cell, Olympus).

Gels (Papers II, III and V)

For drug administration to the middle ear, candidate vehicles with a high viscosity, for simplicity called gels in this summary, were investigated in Paper II. These gels were composed of the polymers sodium carboxymethyl cellulose (0.5% w/w) (NaCMC), sodium hyaluronate (0.5% w/w) (Na HYA), and poloxamer 407 (25% w/w) (POL), respectively. For semi-quantification of the elimination of the vehicles from the middle ear cavity the vehicles were mixed (5:95, v/v) with coal suspension.

A hyaluronan (0.5% w/w) gel containing gadolinium-diethylenetriamine pentaacetic acid-bis methylamine (Gd-DTPA-BMA) at a concentrati on of 2% w/w was used in the studies on which Paper III is based. For the *in vitro* release studies in this paper a gel composed of Gd-DTPA-BMA (2% w/w) in hyaluronan

(0.5% w/w) and carbopol (1% w/w) was prepared as well as the same gel without hyaluronan, as a reference.

A thiosulfate-containing (0.10 M) hyaluronan (0.5% w/w) gel was used for the *in vivo* studies of Paper V. A control gel without sodium thiosulfate was also prepared. For the *in vit*ro studies in this paper the gels also contained carbopol (1% w/w) and a reference gel containing thiosulfate (0.10 M) in carbopol (1% w/w) was also prepared.

A phosphate buffer (about pH 7.2) containing NaCl (9 mg/ml) was used for preparation of all the gels described above. All vehicles were autoclaved before being administered *in vivo* to the animals. The osmolality of the thiosulfate-containing (0.10 M) hyaluronan (0.5% w/w) gel was about 340 mOsm/kg, which is slightly higher than that of blood and inner ear fluids (Sterkers et al., 1984).

Rheological measurements (Paper II)

Rheology is the study of materials with both solid and fluid characteristics and is commonly used for characterizing polymer solutions or gels. According to a rheological definition a gel is a material in which the elastic properties dominate over the viscous properties (Almdal et al., 1993; Ross-Murphy and McEvoy, 1986). The rheological properties of the three gels of Paper II (NaCMC, NaHYA and POL) were investigated using a Bohlin VOR Rheometer (Bohlin Reologi, Lund, Sweden), a controlled rate instrument of the couvette type (Bohlin, 1988). Prior to the measurements the gels were transferred to a concentric cylinder (C8) and centrifuged to remove entrapped air. Silicon oil was added to the surface to prevent evaporation. Viscosimetric measurements were then carried out on NaCMC and NaHYA at 37°C, and oscillating measurements were performed on POL at 20, 25, 30, 35, and 40°C.

Drug release studies (Papers III and V)

The drug release studies were performed using a modified USP paddle method. The set-up used was a Pharma Test PTWIIUSP bath (Pharma Test Apparatebau, Germany) incorporating six

beakers in which custom-made sample containers could be immersed. The sample container was filled with the different gels and then covered with a coarse-mesh plastic net, with the purpose of hindering diffusion of the polymers, followed by a stainless steel net to prevent gels from expanding or changing shape in the container. Finally the container was screwed together with its top section.

The gel containers were immersed in 300 ml drug release medium, usually comprised of NaCl (9 mg/ml). The volume was chosen so that the absorbance for each drug remained at detectable levels throughout the entire experiment and care was taken to make sure of all the release studies were performed under sink conditions.

With the help of a peristaltic pump and ismaprene tubing (Ismatec SA, Zürich, Switzerland), the receiving medium was continuously pumped through a UV-vis spectrophotometer (Schimadzu UV-1601, Schimadzu, Kyoto, Japan). The absorbance was measured automatically at regular intervals. Three measurements were performed on each gel and the absorbance data was used to approximate the Fickian diffusion coefficient for each experiment.

Local administration of gels to the middle ear cavity (Papers II, III and V)

Local administration of the different gel formulations (Papers II, III and V) was performed by injection to the auditory bulla through the skin of the auricle (volume: 0.15 mL; needle: BD Microlance™ 30G, external diameter 0.3 mm). Prior to the injection, a myringotomy of the tympanic membrane was made to evacuate air. An operating microscope was used to determine the point of injection and to observe the flow of the vehicle into the middle ear cavity.

In Paper III, where magnetic resonance imaging (MRI) was used to study the distribution and elimination of a hyaluronan gel, two additional routes of injection were employed. By using a catheter for injection to the middle ear it was possible to compare

the intensity of the middle and inner ear before and after injection without having to move the animals out of the MRI scanner and recalibrate. In both these groups of animals the gel was injected through a catheter entering the middle ear cavity through a hole drilled in the auditory bulla. In one group a myringotomy was made in the tympanic membrane prior to injection and in the other group the tympanic membrane was left intact.

Visual semi-quantification of coal-marked vehicle by opening the bulla (Paper II)

The three different candidate gels for middle ear administration investigated in Paper II, NaCMC, NaHYA, and POL, were marked with coal. After middle ear administration of the coal-marked gels the animals were allowed to wake up. They were reanesthetized one, two, or three weeks after the administration and the auditory bullae were opened, using a dorsolateral approach. All ears were carefully examined in the dissecting microscope for remnants of the coal-marked vehicles and signs of inflammatory response in the middle ear. Quantification was performed with a four-graded scale, shown in Table 1.

Stage	Criteria
A	No visible signs of NaCMC, NaHYA, POL, or coal; no visible signs of inflammation.
B	Visible coal remnants in the middle ear mucosa; no visible signs of NaCMC, NaHYA, POL, or liquid in the middle ear cavity, around the ossicules, or at the round window; no edema in the middle ear mucosa.
C	Visible coal remnants around the ossicules and/or at the round window; edema in or reddened middle ear mucosa or liquid in the middle ear cavity.
D	Middle ear filled with NaCMC, NaHYA, or POL marked with coal.

Magnetic resonance imaging (Paper III)

In the MRI technique a strong magnetic field is used to align the magnetization spin of mainly hydrogen atoms in the tissues. If radio frequency fields are then applied in a certain way the spin of the hydrogen nuclei might be altered or "flipped". When the "flipped" nuclei return to the aligned state a weak radiofrequency signal is emitted at the resonance frequency. The resonance frequency is proportional to the magnetic field strength where the atoms are situated. The strength of the signal is proportional to the number of atoms excited and returning to aligned state, i.e. the amount of water in the tissue. An image can be constructed if the magnetic field is systematically varied regarding strength and alignment inside the body during the data acquisition. The standard basic scans are the T1-weighted scans in which fat is bright and water dark. These scans are produced using a gradient echo sequence with short echo time and short repetition time. MRI in Paper III was performed using a 4.7 T MRI system (Bruker Medizintechnik GmbH, Karlsruhe-Ettlingen, Germany) with a bore diameter of 400 mm, a shielded 200 mT/m gradient system (Bruker BGA-12) and a circular coil with a diameter of 72 mm. Three-dimensional (3D) MR images were recorded using the spin echo rapid acquisition with relaxation resolution technique (Hennig et al., 1986). A recovery time that yielded a high resolution T1-contrast was selected. Paravision 4.0 (Bruker) software was used for post-processing of the images.

Systemic administration of cisplatin and thiosulfate (Papers IV and V)

Thiosulfate (0.20; 3.3 ml/kg b.w.; mean infusion time: 22 s; Paper IV) and cisplatin (1 mg/ml; 8 mg/kg b.w.; mean infusion time: about 3 minutes; Paper V) were administered systemically under direct visual control through a catheter inserted into the internal jugular vein to ensure a reliable, reproducible, and relevant drug administration in the animal studies.

Sampling in vivo (Papers IV and V)

Perilymph samples were aspirated from ST to evaluate the concentration of thiosulfate in Paper IV and V. Sampling was done through a hole drilled in the basal turn of the cochlea, which was accessed by opening the bulla of the animal. A micromanipulator was used to lower a 1-µl syringe into the drilled hole as quickly as possible to avoid leakage of perilymph. One microliter of ST perilymph was then gently aspirated.

Blood samples of about 0.35 ml were drawn through a catheter inserted into the internal jugular vein (Paper IV and V). In Paper IV, blood sampling was performed from the vein contralateral to the vein that was used for drug administration. After each blood sampling, an equal volume of NaCl (9 mg/ml) was given to the animals in order to rinse the catheter and to replace lost fluids.

Samples of cerebrospinal fluid (CSF) were taken immediately after each perilymph sampling (Papers IV and V). The idea was to estimate how a potential CSF contamination of the perilymph samples might have influenced the quantitative analysis of thiosulfate. CSF was aspirated from the cisterna magna using a suboccipital approach. Puncture of the cisterna magna was performed after exposure of the dura mater (Paper IV) or percutaneously (Paper V).

Samples of the thiosulfate-containing hyaluronan gel formulation were aspirated with a syringe after opening of the auditory bulla, two and three hours after injection to the middle ear. The samples were then analyzed to determine the remaining concentration of thiosulfate in the gel.

Thiosulfate analysis (Papers IV and V)

Thiosulfate in samples from perilymph, blood, CSF and hyaluronan gel (Paper IV and V) was derivatized with the reagent monobromobimane prior to analysis using liquid chromatographic separation on a self-packed strong anionic exchange column with a mobile phase of succinate buffer (36 mM; pH 5.0) and acetonitrile (2:1, v/v) and fluorescence detection (λ_{ex}=excitation wave length:

396 nm; emission wave length: 476 nm). The method was adapted from a study found in the literature (Togawa et al., 1992).

Surface preparations (Paper V)

Surface preparations for hair cell counting were used in the otoprotection study of Paper V to evaluate the extent of cisplatin-induced ototoxicity. Cochleae were fixed by perfusion and bone was dissected away. The cell cytoskeletons were then stained with a fluorescence dye connected to the fungal poison phalloidin. Under a fluorescence microscope (Zeiss-Axioplan) the hair cell loss was assessed semi quantitatively and compared to a historical normative material. The absence of stereociliary bundles and scar formation were criteria for hair cell loss.

Statistics

The Wilcoxon signed-rank test was used to compare repeated measurements on the electrophysiological thresholds (Paper II) and to calculate approximate 95% confidence intervals for median difference in hair cell loss between the control ear and the thiosulfate-treated ear (Paper V). Two-tailed Mann–Whitney test was used to compare two independent groups (Paper V). To compare more than two groups, Kruskal–Wallis (Paper II and V) and Friedman test (Paper V) were employed for independent and dependent groups, respectively. Dunn's multiple comparison test was employed to see which groups differed from which other groups (Paper II and V). Fisher's exact test was used to test if the uptake of the contrast agent in the inner ear was significantly related to injection technique in Paper III.

RESULTS

The round window membrane (Paper I and II)

In all eight ears from the six monkeys examined in Paper I, the rim of the RWM displayed a rich network of capillaries, lymph channels, and sinusoidal veins frequently containing white blood cells. Free cells including macrophages and plasma cells were observed in the loose tissue. In two ears from two different animals, the RWM displayed conspicuous glandular formations, which were harbored in the loose connective tissue of the mucosal layer near the bony insertion of the RWM. The gland-like structures found in these primates seemed to actively secrete mucous material into the middle ear lumen and external surface of the RWM (Figure 4).

The thickness of the RWM in normal, unexposed control guinea pig ears in Paper II was close to 10 µm, which correlated well with a previous study (Saber et al., 2009).

Studies of vehicle (Paper II, III and V)

Rheological measurements in vitro (Paper II)

The rheological investigations showed that both NaCMC and NaHYA should be considered as highly viscous solutions rather than gels. At the shear rates studied, NaCMC thinned in response to shear forces, whereas NaHYA did not. POL formed a semisolid gel and its rheology changed drastically at a temperature of about 15°C. POL thus also fulfilled the rheological definition of a gel, which entails having a frequency-independent elastic (G') modulus and a much higher elastic than viscous (G'') modulus over a large frequency range.

Drug release studies (Paper III and V)

In vitro studies

Gd-DTPA-BMA and thiosulfate diffused quickly out of the matrixes in the *in vitro* studies of Paper III and V; the diffusion rates were independent of the presence of HYA with a mean diffusion

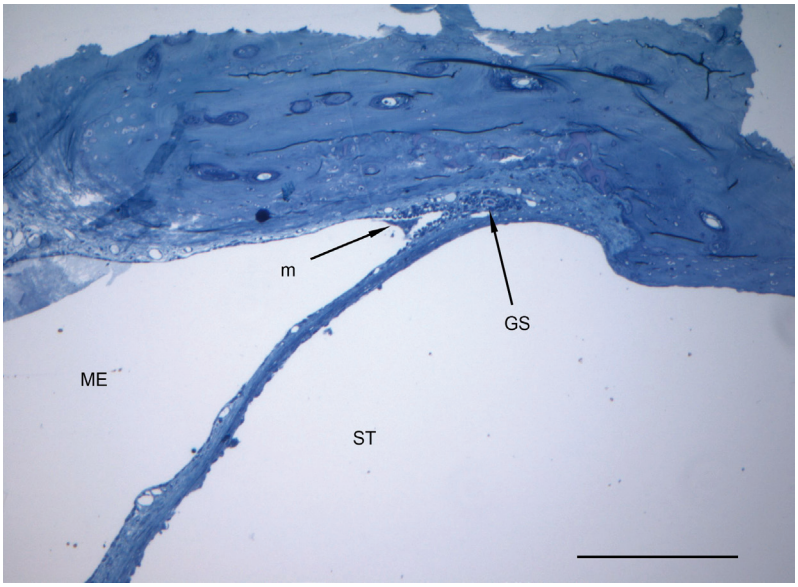

Figure 4. Light microscope view of the round widow membrane in one of the two cynomolgus monkeys displaying conspicuous glandular formations at the rim of the round window membrane. These gland-like structures (GS) were actively secreting mucous material (m) into the middle ear (ME) lumen and external surface of the round window membrane. Scala tympani (ST) (bar = 400 mm)

coefficient (±SD) 2.35 10^{-7} ±2.2 10^{-8} cm^2/s for Gd-DTPA-BMA and 9.57 10^{-6} ± 0.21 10^{-6} for thiosulfate. After a few hours most of the Gd-DTPA-BMA and thiosulfate had left the formulation. This tells us that the HYA formulation does not impede the release of either Gd-DTPA-BMA or thiosulfate and indicates that this polymer does not reduce the uptake speed of a drug when the gel is applied in the middle ear cavity.

In vivo studies

The HYA gel containing Gd-DTPA-BMA showed constant signal intensity during the first three hours of measurements by MRI in Paper III.

After one hour (1-h gel group) and three hours (3-h gel group) most of the thiosulfate remained in the HYA gel aspirated from the middle ear in the studies on which Paper V is based. The

median thiosulfate concentrations were 99 mM (range 31–120 mM, n = 8) and 90 mM (range 75–110 mM, n = 6) in the 1-h and 3-h gel groups, respectively. The difference between the groups was not significant.

Distribution and elimination of the three vehicles after injection to the middle ear (Paper II and III)

The visual semi-quantification of gel elimination *in vivo* (paper II) showed that NaHYA was eliminated from the middle ear faster than NaCMC and POL. In NaHYA-injected ears, only coal remnants were found in the middle ear three weeks after injection. In the other two groups there were also signs of inflammation and gel remnants in the middle ear at three weeks.

In Paper III, the best filling of the middle ear was achieved by the injection technique where the Gd-DTPA-BMA-containing HYA gel was delivered to the middle ear through a percutanous injection through the auditory bulla after a small incision had been made in the tympanic membrane. When this was done, the gel covered the cochlea, including the region of the round

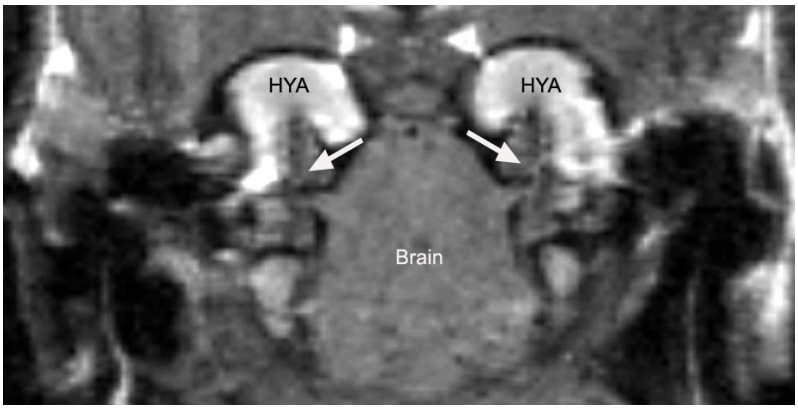

Figure 5. Hyaluronan gel (0.5% w/w) containing a paramagnetic contrast agent injected to both the right and left middle ear by a percutanous injection through the auditory bulla of guinea pig. The middle ears were almost completely filled with HYA gel, which was covering the cochleae (arrows) and the region of the round window membrane at the base of the cochlea.

window niche (Figure 5). The gel continued to cover the cochlea at 24 h in 9 out of 12 examined ears and at 48 h in 4 out of 9 examined ears. The two ears imaged at 72 h and one at 96 h showed gel remnants only at the base of the cochlea and not in the region of the round window niche. Ears injected without an incision in the tympanic membrane displayed an immediate uptake of Gd-DTPA-BMA in the inner ear, which is taken as a sign of rupture of the round window membrane.

Effects of the three vehicles on middle and inner ear (Paper II)

In the studies on which Paper II is based, ABR assessment revealed normal electrophysiological hearing thresholds in ears injected with NaHYA at one, two or three weeks after injection to the middle ear. Conversely, ears injected with NaCMC and POL showed threshold shifts throughout the observation time. This indicates that the negative effect of NaHYA on sound transmission is eliminated faster than the effects of the two other gels. The electrophysiological hearing thresholds shifts of the

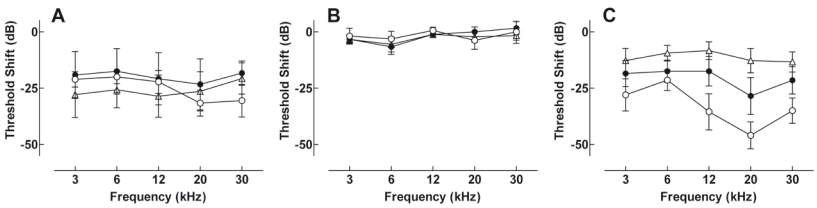

Figure 6. Guinea pigs were subjected to an intratympanic injection (0.15 mL) of sodium carboxymethyl cellulose (0.5% w/w) (A), hyaluronan (0.5% w/w) (B), or poloxamer 407 (25% w/w) (C). Acoustically evoked auditory brainstem response (ABR) assessment was performed before as well as one, two, and three weeks after the injection. The graphs show the electrophysiological hearing threshold shifts, i.e. the difference between the hearing thresholds obtained before and after (one week, open circles; two weeks, closed circles; three weeks; open triangles) the injection. Data are expressed as mean± SEM. The measured frequencies, 3, 6, 12, 20, and 30 kHz, are center frequencies of the presented auditory stimuli.

three groups of guinea pigs after injection of the different gels to the middle ear are shown in Figure 6.

Due to technical problems in the morphological study of Paper II, few observations could be done. Moreover, the number of successful observations differed between the three groups, making it difficult to draw any significant conclusions. However, no remnants of the vehicle and no signs of mucosal inflammation were found in the ears injected with NaHYA, whereas vehicle remnants and a swollen middle ear mucosa was found in animals injected with NaCMC. The mucosa was also swollen in POL-injected ears but no remnants of the vehicle could be seen. There were no signs of damage to the hair cells or SV in the basal turn the cochlea, close to the RWM in any of the ears that were examined.

Drug distribution to the inner ear (Paper IV and V)

Systemic administration of thiosulfate (Paper IV)

Thiosulfate was rapidly and extensively distributed to the perilymph of the ST after i.v. administration. The area under the median concentration–time curve of ST perilymph and blood ultra-filtrate was 3100 µM x min and 6300 µM x min, respectively. The elimination of thiosulfate from perilymph of the ST was slower than that from blood, resulting in higher concentrations of thiosulfate in perilymph at the end of the observation period, three hours after injection (Figure 7).

The thiosulfate concentrations in CSF were well below those in ST perilymph and blood ultrafiltrate at the same target times.

Local administration of thiosulfate (Paper V)

The median concentrations of thiosulfate in ST perilymph after local administration of a thiosulfate-containing HYA gel to the middle ear of the guinea pig in Paper V were 137 µM (range 14.5–312 µM, n=7) and 148 (range 57.4–912 µM, n=7) in the 1-h and

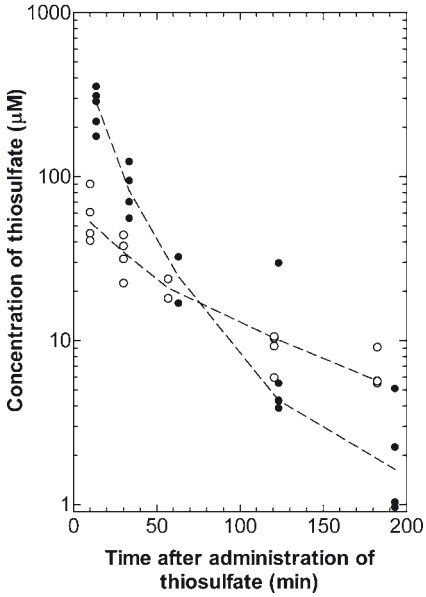

Figure 7. Concentrations of thiosulfate in blood (closed circles) and scala tympani perilymph (open circles) of guinea pigs after an i.v. bolus injection of thiosulfate (0.20 M; mean injection volume: 1.28 ml). Each symbol represents one sample. The broken lines connect the median concentration of thiosulfate at each sampling time point in blood as well as in perilymph.

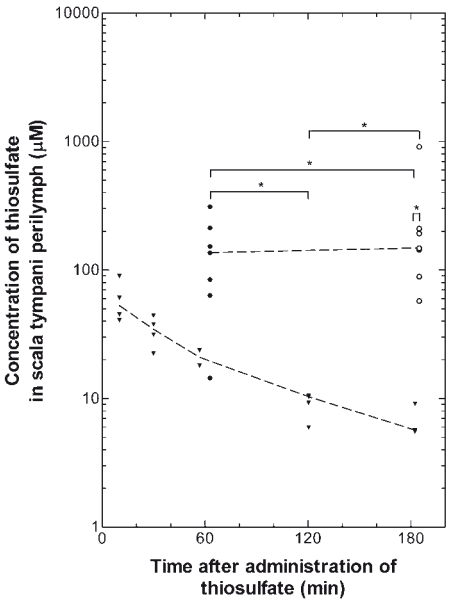

Figure 8. Concentration of thiosulfate in scala tympani perilymph of guinea pigs after an i.v. bolus injection of thiosulfate (0.20 M; men injection volume: 1.28; filled triangles) and intratympanic injection (0.10 M in hyaluronan (0.5% w/w) gel; mean injection volume: 0.17 ml; open and closed circles). The gel was removed after one hour (closed circles) or three hours (open circles) prior to perilymph sampling. Each circle and triangle represents one sample. The thiosulfate concentrations in scala tympani perilymph were significantly higher (indicated with a star) in the gel groups compared to the i.v. group 120 and 180 minutes after the i.v. administration. The broken lines connect the median concentrations of thiosulfate at each sampling time point in the i.v. group as well as in the gel groups.

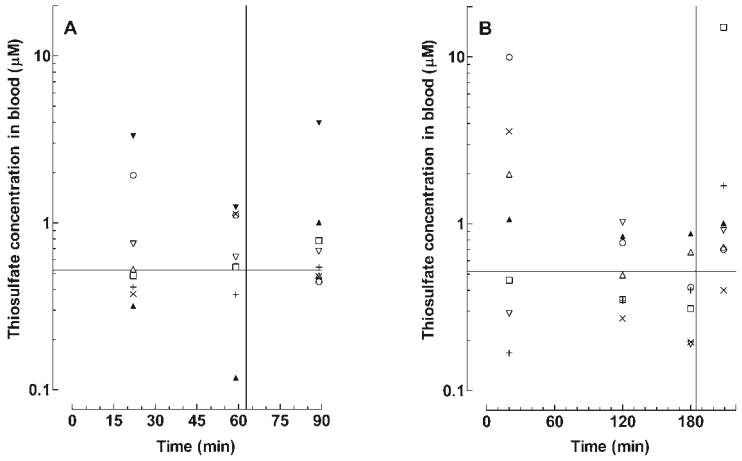

Figure 9. Concentrations of thiosulfate in blood of guinea pigs treated with a thiosulfate-containing (0.10 M) hyaluronan (0.5% w/w) gel (mean injected volume: 0,17 ml) applied in the middle ear for one hour (1-h gel group; A) or three hours (3-h gel group; B) Each symbol represents one animal and each animal was sampled three times in the 1-h gel group and four times in the 3-h gel group. The solid horizontal lines show the approximate endogenous thiosulfate concentration in the guinea pig and the solid vertical lines indicate the mean time of removal of the gel.

3-h gel groups, respectively. No significant difference was found between the two groups.

Figure 8 shows the concentrations of ST perilymph from the 1-h gel group, the 3-h gel group (Paper V) and the group treated with thiosulfate i.v. (Paper IV). The local administration system gave a significantly higher concentration of thiosulfate in ST perilymph of the basal turn compared to the i.v. administration, even though the amount of thiosulfate given locally was only 7% of the i.v. dose.

The thiosulfate concentration in blood after intratympanic administration remained low during the entire study in both the 1- and 3-h gel groups as shown in Figure 9. There was no significant difference between the 1-h gel group and the 3-h gel group. The thiosulfate concentration was low in CSF samples taken at the end

of the experiment. The median concentration of thiosulfate was 0.80 µM (range 0.67–0.86 µM, n=6) in the 1-h gel group and 0.81 µM (range 0.70–1.0 µM, n=6) in the 3-h gel group.

Otoprotective effect of a locally administered thiosulfate-containing gel against cisplatin induced ototoxicity (Paper V)

A protective effect against cisplatin-induced ototoxicity obtained by middle ear application of thiosulfate (0.1 M) in HYA gel three hours prior to cisplatin administration (8 mg/kg b.w., i.v.) was confirmed. Ten out of eleven animals with damaged control ears exhibited only minor OHC loss in the ear treated with thiosulfate-containing HYA gel. No inner hair cell loss was seen in any of the ears. Cytocochleograms from cisplatin-treated guinea pigs, representing the median OHC loss in control ears and in ears treated with a thiosulfate-containing HYA gel prior to cisplatin treatment are shown in Figures 10A and 10B, respectively.

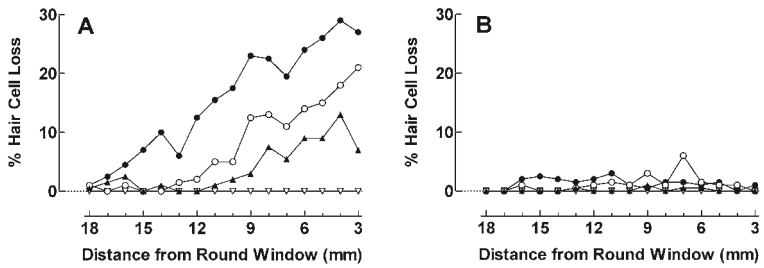

Figure 10. Cytochocleograms for cisplatin-treated guinea pigs (8 mg/kg b.w., i.v.) showing loss of outer hair cells (OHCs) in control ears (A) and ears treated with a thiosulfate containing (0.10 M) hyaluronan (0.5% w/w) gel (B). The gel was injected to the middle ear cavity three hours prior to administration of cisplatin. Inner hair cells (IHCs) are represented by open triangles and OHCs in the first row by filled circles, in the second row by open circles and in the third row by closed triangles. Data are expressed as median values.

The difference in OHC loss between the control and the thiosulfate-treated ears of each animal was calculated and the median values are presented in Figure 11. The difference observed in the basal turn of the cochlea was considered significant as the approximate 95% confidence interval did not overlap, y=0.

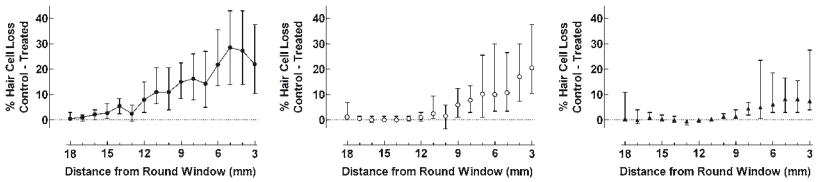

Figure 11. Cytocochleograms for cisplatin-treated guinea pigs (8 mg/kg b.w. i.v.) showing difference in loss of outer hair cells (OHCs) between control ears and ears treated with a thiosulfate-containing (0.10 M) hyaluronan (0.5% w/w) gel. The gel was injected to the middle ear cavity three hours prior to administration of cisplatin. The closed circles, open circles, and closed triangles represent the OHs in the first, second, and third rows, respectively. Data are expressed as medians with approximate 95% confidence intervals.

DISCUSSION

Therapeutic management of inner ear disease is an expanding scientific field. Advances in molecular biology and especially in the basic understanding of mechanisms associated with inner ear disease will lead to new approaches for treatment of the large group of patients who suffer from inner ear disorders. The cochlea, embedded in the petrous part of the temporal bone, is a particularly challenging target for pharmacological treatment and new technologies will be required to provide safe and efficacious delivery of medication to the inner ear. It was hypothesized at the start of this doctoral project that local administration of an antioxidant to the middle ear provides protection against an ototoxic injury. A fundamental idea was that intratympanic drug delivery circumvents some of the problems associated with systemic delivery, such as drug interaction and systemic side effects.

The local delivery systems used in this thesis were high viscosity formulations injected to the middle ear, serving as vehicles for drugs aimed for inner ear treatment. The main and chronologically last finding was that thiosulfate-containing (0.1 M) HYA gel (0.5% w/w) injected to the middle ear three hours prior to i.v. injection of cisplatin (8 mg/kg) protected against OHC loss in the guinea pig. Prior to this final study presented in paper V various aspects of local drug delivery to the inner ear were first elucidated.

Round window membrane – a route for drug delivery

First, the morphological and immunological properties of the RWM were investigated. This membrane is believed to be the primary route for drug transport from the middle to the inner ear. The experiments aimed to provide deeper understanding of mechanisms that might influence this transport. The study on which Paper I is based was performed on the cynomolgus monkey, a primate closely related to the human being. A rich network of capillaries, lymph channels, and sinusoidal veins frequently containing white blood cells were displayed at the rim of the

RWM. In the loose connective tissue free cells, including plasma cells and macrophages, were identified. Conspicuous glandular formations at the bony insertion of the RWM were observed in one third of the animals. Such formations have not previously been described in the literature. The findings suggest that a local defense system is housed within the rim of the RWM. This could explain why labyrinthitis is rare, even though the middle ear is an area prone to infections (Hyden et al., 2006; Kitsko and Dohar, 2007). A local immunodefense system in the inner ear has earlier been suggested, with the endolymphatic sac playing a central role (Couloigner et al., 2004; Garcia Berrocal and Ramirez-Camacho, 2000; Harris and Ryan, 1995; Harris et al., 1997). Still the RWM most probably represents one of the major pathways for possible transport of toxins and infectious material from the middle ear into the ST and inner ear. Moreover high-frequency SNHL both in animals and patients with otitis media strongly suggest preferential involvement of the cochlear basal turn (Cureoglu et al., 2004; Joglekar et al., 2010; Paparella et al., 1980; Schachern et al., 1992). The local immunodefense system appears to involve mucin secretion from goblet cells and specific action via white blood cells, including monocytes/macrophages acting as a first line of defense. The second line of defense most probably entails integrated, specific functions involving immunocompetent cells (e.g., plasma cells) with antigen trapping and processing, leading ultimately to degradation and detoxification of the invader or invading material. These mechanisms all involve the cascades of specific immunoreactions with complex interactions between cell components, cell trafficking, and local humoral interaction. During a mild inflammatory response induced by injection of NaCl (9 mg/ml) to the middle ear, the mucosa was found to be slightly swollen at the RWM towards the middle ear. At the same time, the number of free cells within the subepithelial space in the round window niche increased, as did the number of white blood cells in the blood vessels. An increased thickness of the RWM was also seen in the saline-injected ears of guinea pigs in Paper II, strengthening the impression of a locally developed

immunodefensive system at the round window. The inflammatory alterations localized to the RWM seen in both the cynomolgus monkey and the guinea pig indicate that permeability of the RWM may undergo dynamic changes in response to instillation of exogenous products to the middle ear cavity.

An activation of the immunodefense system of the RWM could affect the membrane's transport properties in several ways; such changes would also influence drug delivery when a drug is applied locally to the middle ear for treatment of the inner ear. This might explain some of the inter-individual variability in drug uptake into the inner ear after application in the middle ear and at the RWM (Hahn et al., 2006; Mikulec et al., 2008). Thickening and microscopic signs of inflammation in the RWM have been observed when drugs and vehicles have been applied to the RWM or intratympanically (Nordang et al., 2003; Saber et al., 2009). Drug solution osmolarity, benzyl alcohol content and possible drying of the RWM during suctioning of the middle ear, have all been shown to influence the permeability of the RWM (Mikulec et al., 2008). It has been suggested that manipulations of the RWM that initially increase permeability, subsequently result in thickening and scarring (Goycoolea, 2001) that could reduce permeability for later attempts at inner ear drug delivery. Injury to the local defense system by manipulation may make the inner ear more susceptible to toxins and direct spread of microorganisms that are present in a non-sterile middle ear space. All these factors may influence the drug transport to the inner ear from the middle ear and need to be taken into consideration when developing new technologies for drug delivery.

Vehicles for drug delivery to the inner ear

The second study (Paper II) studied the rheological properties of three different high viscosity formulations aimed for intratympanic drug administration: NaCMC, NaHYA (called HYA in the following papers III and V) and POL. Their safety was also assessed.

Sodium hyaluronate, which was used for preparation of NaHYA, is the sodium salt of hyaluronic acid. In the literature, hyaluronan

is used as a general term for this polymer, irrespective of its degree of dissociation (Balazs et al., 1986). Hyaluronan occurs naturally in humans, particularly in the synovial fluid, the umbilical cord, the nasal cartilage, and the vitreous body (Laurent and Fraser, 1992). It has also been found in the inner ear (Friberg et al., 1989). Locally applied exogenous hyaluronan has been used for a long time in otological research, both in experimental animals (Angeli et al., 2007; Anniko et al., 1987; Bagger-Sjoback, 1991; Bjurstrom et al., 1987; Dogru et al., 2009; Krupala et al., 1998; Laurent et al., 1991; Saber et al., 2009) and humans (Angeli et al., 2007; Arriaga and Goldman, 1998; Bagger-Sjoback et al., 1993; Engstrom et al., 1987; Gouveris et al., 2005; Selivanova et al., 2005; Stenfors, 1987). These studies have shown that hyaluronan is well tolerated and not ototoxic, even when administered into the middle ear under conditions that have allowed direct access to the inner ear (Bagger-Sjoback, 1991; Laurent et al., 1991). The light microscopic investigation described in Paper II showed that NaHYA induced less thickening of the RWM and TM than the other two vehicles, NaCMC and POL. The effect of NaHYA on the RWM agrees with that of a previous study, in which a similar hyaluronan vehicle was used to study neomycin transport from the middle ear to the inner ear after intratympanic injection (Saber et al., 2009). In the study of Saber et al., the thickness of the RWM returned to normal values after four weeks. Exogenous hyaluronan is reported to be eliminated from the middle ear cavity primarily via the Eustachian tube (Laurent et al., 1986). It may be totally eliminated within a day in the rat, according to an experimental study using a vehicle with a hyaluronan concentration twice that of the present investigation (Laurent et al., 1986). However, results of the semi-quantitative study (paper II) using NaHYA marked with coal indicate that a hyaluronan-based vehicle may stay in the middle ear considerably longer in the guinea pig. An important finding was that NaHYA did not induce any significant shifts in the electrophysiological hearing threshold one, two, and three weeks after administration of NaHYA. Neither were any remnants of NaHYA in the middle ear found in the light microscopic investigation six days after its

administration to guinea pigs (paper II). In the present project, the hyaluronan concentration was not varied, but it is well known that the viscosity of a hyaluronan gel is directly proportional to its concentration (Lapcik et al., 1998). Even though this was not examined it is probable that the elimination rate of a hyaluronan vehicle from the middle ear cavity would be reduced when the concentration of hyaluronan is increased.

Sodium carboxymethyl cellulose is a widely used additive in different pharmaceutical formulations, mostly as a viscosity modifier, but also as an emulsifier (Wade and Weller, 1994). It is also used in artificial teardrops. In the present study, NaCMC was found to be pseudoplastic, which means that the carboxymethyl cellulose molecules were deformed and realigned in the streamlines of flow, resulting in a decrease in the viscosity. This behavior occurs when a pseudoplastic formulation is forced through the needle of a medical syringe, which facilitates injection. In contrast, NaHYA did not exhibit a shear thinning behavior. Nonetheless, hyaluronan has been shown to be pseudoplastic when a higher shear force is applied or when it is less concentrated (Lapcik et al., 1998). Sodium carboxymethyl cellulose is considered nontoxic and non-irritant, and is employed also as a food additive (Wade and Weller, 1994). However, in contrast to hyaluronan, there are few published studies on the use of sodium carboxymethyl cellulose in otological research. The results of the present study show that NaCMC might be less convenient for intratympanic administration than NaHYA. In fact, sodium carboxymethyl cellulose might even be ototoxic, according to recently published data from a guinea pig study (Antonelli et al., 2010). However, that study used a commercial formulation (Sinu-Foam) intended for nasal application and it is unknown whether the hearing loss was caused by sodium carboxymethyl cellulose itself or by some other component or property of the formulation.

Poloxamer 407 is a polyoxyethylene block copolymer used in several different pharmaceutical preparations, both for internal and topical use. Poloxamers respond to changes in temperature, being less viscous when refrigerated or at room temperature than

at body temperature. To facilitate the intratympanic injection in the present study, samples of POL were taken from the refrigerator immediately prior to the injection. Despite this precaution, the viscosity of POL was still high, and to avoid excess trauma to the TM, POL (as well as NaCMC and NaHYA) was injected through the auditory bulla. Similar to sodium carboxymethyl cellulose and hyaluronan, poloxamer 407 is considered nontoxic and non-irritant (Wade and Weller, 1994). Nonetheless, the results of the present study imply that POL is less suitable for intratympanic administration than NaHYA. In particular, POL induced a striking thickening of the TM and had substantial effects on the electrophysiological hearing thresholds in the guinea pig. However, according to recent publications, poloxamer 407 may also be well tolerated when administered into the middle ear (Piu et al., 2011; Wang et al., 2009). In one of the cited studies, most of the conductive hearing loss had disappeared about four weeks after intratympanic administration of poloxamer 407 (Piu et al., 2011), and several weeks earlier in two other studies (Salt et al., 2010; Wang et al., 2009). These studies used a lower concentration of poloxamer 407 (16% and 17% versus 25% in our study) and a smaller volume was injected (50 µl versus 150 µl in our study), which might explain the more promising results in the other studies as compared to those presented in this thesis.

The overall results of paper II speak in favor of NaHYA. Especially, in contrast to NaCMC and POL, NaHYA did not cause any obvious prolonged conductive hearing loss as determined by ABR. The results of the elimination and morphological investigations support the conclusion that NaHYA is the most promising candidate for intratympanic administration. NaHYA was therefore selected for further investigations. In the following discussion NaHYA will be called HYA gel.

In vivo study of HYA gel distribution and elimination using magnetic resonance imaging

A major concern with local administration of a drug to the inner ear using the middle ear route is the adherence of the vehicle or

solution to the RWM. The literature offers no detailed information about the residence time of a vehicle in the round window niche after intratympanic administration. In order to get a more clear view of middle ear filling after intratympanic injection, different techniques for visualization of the HYA gel were discussed before the study in paper III was designed. Moreover, it was also of great interest in this phase of the doctoral project to establish the optimal technique for injection of HYA gel to the middle ear of the guinea pig. Experimental MRI was found to be a suitable tool to explore in vivo how a vehicle behaves over time in the middle ear after an intratympanic injection. Therefore, this in vivo study compared HYA gel distribution and elimination in the middle ear cavity after use of three different techniques for intratympanic injection. The paramagnetic contrast agent Gd-DTPA-BMA was added to HYA gel which was then injected to the middle ear of three groups of guinea pigs and investigated using high field experimental MRI. The 4.7 T MRI system, using a T1-weighted 3-dimensional rapid acquisition with relaxation enhancement sequence, provided a good visualization of the middle ear and cochlea. This non-invasive method made it possible to get a better understanding of how HYA gel filled the middle ear. In all three animal groups the gel was well distributed and filled the middle ear cavity completely, covering the major portion of the cochlea and most important the region of the round window niche. In the in vitro preparation approximately 70% of the Gd-DTPA-BMA had disappeared from the gel within three hours whereas in vivo the intensity of marker in the gel did not decrease during the first three hours of observation following intratympanic injection. A possible explanation for this observation could be that the middle ear in this context can be regarded as a semi-closed compartment, in contrast to the open conditions in the basins of the in vitro study. In this perspective the middle ear could be seen as a reservoir for drug transport to the cochlea after intratympanic injection of drug-loaded HYA gel. From a clinical standpoint it is important to establish that a drug can diffuse freely in and out of the gel and that the gel does not retain it in the middle ear cavity. Under in

vivo conditions the surrounding mucosa in the middle ear will most probably be rapidly saturated with the drug and therefore the gel will continue to have a high concentration, allowing passage over the RWM for a longer period of time. In the present study one could still observe gel with Gd-DTPA-BMA signaling in remnants at the base of the cochlea but not in the region of the round window niche up to 72 h after injection. This means that the residence time of HYA gel in the round window niche was shorter than 72 hours, indicating that the effective release of a drug from the HYA gel to the inner ear would not exceed 72 hours. If local treatment methodologies are to be developed for use in human patients, gel residence times in the human round window niche should be evaluated in similar clinical MRI studies.

Injection techniques

The most reliable minimally invasive method for injection to the middle ear of the guinea pig was a procedure involving HYA gel injection through the skin of the auricle after an incision in the upper quadrant of the tympanic membrane. This method allowed air to escape as pressure increased and the middle ear was filled with the gel in a predictable way. Thus, a true transtympanic injection through the tympanic membrane was not used in the following study. As a result of earlier studies on experimental animals and on patients it is generally accepted that the RWM is permeable to Gd-DTPA-BMA and gadolinium-tetra-azacyclo-dodecane-tetraacetic acid (Counter et al., 1999; Counter et al., 2000; Duan et al., 2004; Zou et al., 2010a; Zou et al., 2010b) (Yoshioka et al., 2009) (Nakashima et al., 2007; Nakashima et al., 2009). The concentration of Gd-DTPA-BMA in the HYA gel (10 mmol/l) used in the current study was selected for visualization of the gel in the middle ear and not primarily for observation of Gd-DTPA-BMA uptake in the inner ear. In studies where Gd uptake in the cochlea has been shown, considerably higher concentrations of Gd-DTPA-BMA and the other gadolinium compound have been used (Duan et al., 2004) (Zou et al., 2010a) (Nakashima et al., 2007; Nakashima et al., 2009; Yoshioka et al., 2009). It is therefore most

probable that the lack of cochlear uptake seen in groups 2 and 3 in which myringotomy was used before injection was due to the low concentration of Gd-DTPA-BMA. In contrast, in group 1, which was given Gd-DTPA-BMA-containing HYA gel with an intact tympanic membrane, Gd-DTPA-BMA was immediately taken up into ST, indicating a perforation of the RWM. These data suggest that an injection technique without paracentesis of the TM can be hazardous to the RWM and likely also to cochlear function.

Drug transport to the inner ear

Paper II and III identified the HYA gel as a promising vehicle for middle ear administration of drugs aimed for inner ear treatment. The next step would be to investigate if a drug could actually be delivered to the inner ear in measurable concentrations using the HYA gel in the guinea pig model. Our research group has long experience of research on cisplatin-induced ototoxicity and our collaborators at the Karolinska Pharmacy had recently identified the endogenous ion thiosulfate (Figure 12) as an interesting candidate compound for protection against cisplatin-induced ototoxicity (Videhult et al., 2006). The next goal was to investigate if locally administrated thiosulfate in HYA-gel could reach the cochlea to a greater extent than after systemic administration and also to establish whether ototoxicity induced by cisplatin could be prevented.

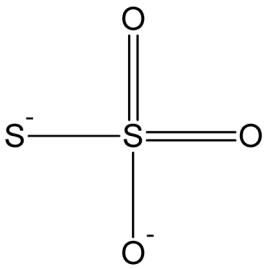

Figure 12. The molecular formula of thiosulfate. A sulfur-containing antioxidant with otoprotective properties.

The results from Paper IV and Paper V show that the highest concentration of thiosulfate in the basal turn of the cochlea was achieved by using the local administration strategy even though the amount of thiosulfate given locally was only 7% of the i.v. dose (Fig 7). The concentration of thiosulfate in ST perilymph of gel-treated guinea pig also remained high for a longer period of time, whereas it decreased rapidly in i.v. treated guinea pigs. This indicates that thiosulfate was stored in the middle ear, prolonging cochlear drug uptake.

Assessment of thiosulfate in ST perilymph is a common method to establish how much of the administered drug has reached the inner ear. However, drug concentrations in perilymph are not necessarily a good measure for inner ear pharmacokinetics. Drug trafficking inside the inner ear is complex and the usefulness of the perilymphatic route might vary for different substances, depending upon their chemical structure. For example an endolymphatic route for gentamicin to cochlear hair cells has been suggested (Wang and Steyger, 2009). Thiosulfate was sampled from the ST by aspiration of 1 µl of perilymph from the basal turn of the cochlea. Sampling from ST in the guinea pig is more straightforward than sampling from SV and SM. Moreover, sampling from the basal turn of the cochlea does not involve as severe a surgical trauma to the animal as sampling from the apex does. This type of sampling can therefore be performed with fewer effects on the homeostasis of the animal, i.e. the samples can be regarded as taken under reasonably physiological conditions. In cisplatin-induced ototoxicity the outer hair cell loss is most pronounced in the basal turn; thus the concentrations of thiosulfate in this part of the cochlea are the most interesting. However, one should always consider the risk of contamination with CSF during perilymph sampling in the guinea pig (Salt et al., 2003) since the open cochlear aqueduct, which connects the inner ear with the CSF-filled space in the central nervous system, is located at the base of the cochlea near the round window. To avoid spillover of perilymph it is important to sample small volumes, not to close to the round window, and the sampling

should be performed quickly (Hara et al., 1989). The 1-µl samples of perilymph that were aspirated from ST as described in Paper IV and V are expected to contain about 20% CSF (Hara et al., 1989). However, the low concentrations of thiosulfate detected in pure CSF samples confirm that the thiosulfate concentrations in ST perilymph were more likely underestimated than overestimated.

The concentrations of thiosulfate in blood after local administration were about the same as those in blood 3 hours after i.v. administration (c.f. Figure 8A and 8B to Figure 6). There was a large inter- and intra-individual variability in the data shown in Figure 8A and 8B but the concentrations of thiosulfate in blood from the gel groups were in most cases similar to the endogenous levels (indicated with solid horizontal line in Figures 8A and 8B). Even in the most extreme cases, when thiosulfate levels reached 15 µM, the risk that the antineoplastic effects of cisplatin might be reduced seems negligible; much higher levels are required for inactivation of cisplatin and MHC (Pierre, 2010).

The in vitro studies of drug release in Paper V showed that practically all thiosulfate had disappeared from the gel within 2 h whereas in vivo there was still a high concentration of thiosulfate when the gel was aspirated from the middle ear 3 h after intratympanic injection. These results are in line with the results of Paper III and are thought to be due to the closed compartment of the middle ear serving as a reservoir for a prolonged drug release. This might be of particular importance for protection against cisplatin-induced ototoxicity, as cochlear injury develops gradually over several days after treatment (Laurell and Bagger-Sjoback, 1991). However, it has been shown that ototoxicity caused by platinum-containing antineoplastic drugs is dependent upon the drugs' pharmacokinetics in the inner ear, particularly on the area under the concentration-time curve (Hellberg et al., 2009). Therefore, the presence of thiosulfate in the cochlea might be most critical when the cisplatin concentration in ST perilymph is at its highest, which occurs during the first hours after a single i.v. injection in the guinea pig (Laurell et al., 1995a). If this is the case, the middle ear administration strategy is also superior to systemic

administration since it offers higher thiosulfate concentration in ST perilymph during the first phase of ototoxicity.

From the results of Paper III and the pharmacokinetic study of Paper V we could conclude that injection of a thiosulfate-loaded HYA gel to the middle ear is a feasible method to obtain high concentrations of thiosulfate in the basal turn of cochlea while blood levels were maintained low. There are undesireable audiological symptoms, such as fullness of the ear, but these are temporary. This local administration strategy also seemed to offer a continous distribution of thiosulfate to the inner ear.

Protection against cisplatin ototoxicity by local administration of a thiosulfate-containing HYA gel

In the final study of Paper V, the efficacy of the thiosulfate-loaded HYA gel against cisplatin-induced ototoxicity was explored. The study design included injection of HYA gel to the middle ear three hours before cisplatin treatment. This was based on the results from the pharmacokinetic study, which showed that the lowest concentrations of thiosulfate in perilymph were higher in the 3-h gel group than in the 1-h gel group even though the median concentrations of thiosulfate in ST did not significantly differ between the groups. Evaluation of OHC loss by the use of surface preparations of cochleae harvested four days after cisplatin administration showed that thiosulfate (0.10 M) in HYA gel (0.5% w/w) protected against cisplatin-induced hair cell loss in the guinea pig. Thus, the results of this doctoral project support the hypothesis that local administration of an antioxidant to the middle ear provides protection against an ototoxic injury.

It remains to be established if this local drug delivery system can be used to apply drugs to the inner ear of patients. The main drawback of this method is most probably the conductive hearing loss that will cause temporary discomfort for the patients. However, the results from Paper II and III indicate that most of the gel was eliminated within a week, and a transient conductive hearing loss might be accepted – at least by patients undergoing high-dose cisplatin treatment – in order to avoid ototoxic side

effects. If the HYA gel were to be used for treatment of ISSHL when the patients are already suffering from a hearing loss, this issue would be even less relevant. When a prolonged time at the site of absorption is desired, a gel or viscous formulation is advantageous due to its mucoadhesive (Edsman and Hagerstrom, 2005) and rheological (Carlfors et al., 1998; Edsman et al., 1998) properties. By varying the concentration of the gel or high viscosity formulation administered to the middle ear, it will be possible to maintain inner ear drug exposure for a desired period of time. Moreover, injection of a drug-loaded HYA gel can be repeated.

CONCLUSIONS

1. The morphological study of the round window in a primate showed existence of a local defense system housed within the rim of the round window membrane (RWM). Gland-like structures harbored in the loose connective tissue of the mucosal layer near the bony insertion of the RWM were identified and seem to be an important part of this local immunodefense of the inner ear
2. Sodium hyaluronate (HYA gel) was found to be a suitable vehicle for middle ear administration aimed for pharmacological treatment of the inner ear. In contrast to sodium carboxy methyl cellulose and poloxamer 407, HYA gel did not cause prolonged elevations of electrophysiological hearing threshold. The results of the elimination and morphological investigations also support the conclusion that HYA gel is the most promising candidate for intratympanic administration.
3. Magnetic resonance imaging showed that HYA gel was distributed in a predictable way and filled the middle ear cavity well after injection into the tympanic bulla in the guinea pig. Myringotomy was needed before the middle ear injection in order to avoid traumatic damage to the round window membrane. The HYA gel remained in close vicinity to the round window membrane for more than 24 h.
4. Thiosulfate was rapidly and extensively distributed to the perilymphatic compartment of the cochlea after an i.v. bolus injection in the guinea pig. The elimination of thiosulfate from perilymph was slower than from blood, resulting in higher concentrations in perilymph than in blood at the end of the observation time, three hours after injection.
5. Although HYA gel did not inhibit the release of thiosulfate in vitro, a high concentration of thiosulfate remained in the HYA gel three hours after middle ear administration, indicating that drug release to the inner ear is prolonged in vivo.
6. Compared to i.v. administration, local administration of thiosulfate by injection of thiosulfate-containing HYA gel to the middle ear cavity resulted in higher concentrations of

thiosulfate in perilymph, while levels in blood remained low, indicating a low risk of interference with the antineoplastic effects of cisplatin.

7. Compared to i.v. administration, local administration of thiosulfate by injection of thiosulfate-containing HYA gel to the middle ear cavity offered a continuous distribution of thiosulfate to the perilymphatic compartment of the cochlea. Since cisplatin-induced ototoxicity develops progressively over several days, this continuous distribution of thiosulfate might help protect the inner ear from damage. This reservoir function of the drug delivery system might also be useful for treatment of other inner ear disorders where a prolonged drug release would be advantageous.
8. Cisplatin-induced outer hair cell loss was prevented by administration of thiosulfate-containing HYA gel to the middle ear in a guinea pig model. This supports the idea that the local drug delivery system using an intratympanic injection of HYA gel as a vehicle might be used to treat inner ear disorders and that future studies on patients are of great clinical interest.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Hörselsjukdom är en av de tio vanligaste sjukdomarna i mellan- och höginkomstländer med cirka 250 miljoner drabbade människor världen över. Under de senaste decennierna har forskningen lett till betydande framsteg genom att på cellnivå förklara bakomliggande mekanismer till innerörats sjukdomar. En av de stora utmaningarna vid läkemedelsbehandling av inneröronsjukdom är innerörats otillgänglighet för läkemedel på grund av olika barriärsystem som hindrar upptag av läkemedel. Dessutom är innerörat svåråtkomligt för en direkt tillförsel av läkemedel eftersom det sitter i skallbasen. Nya tekniker för att leverera läkemedel till innerörat på ett effektivt och säkert sätt skulle vara av stort värde för behandling av patienter med inneröronsjukdom. Genom att ge läkemedel lokalt till innerörat i stället för en behandling med tabletter eller läkemedelsinjektion till blodet skulle man kunna undvika vissa problem såsom läkemedelsbiverkningar och läkemedelsinteraktioner och sannolikt även kunna öka koncentrationen av läkemedlet i innerörat.

Målet i avhandlingen har varit att utveckla en metod för lokalbehandling av innerörat. Metoden bygger på injektion till mellanörat av ett bärarmaterial laddat med läkemedel för transport av läkemedlet till den närbelägna hörselsnäckan i innerörat.

Hörselsnäckans runda fönstermembran är troligen den huvudsakliga transportvägen för läkemedel till innerörat när det ges genom en injektion till mellanörat. I avhandlingens första studie (artikel I) undersöktes spindelapors runda fönster i mikroskop och förekomsten av ett lokalt immunförsvar kunde påvisas. En körtelliknande struktur som tidigare inte är beskriven i litteraturen identifierades i bindväven invid runda fönstermembranets infäste i hörselsnäckans benkapsel. Dessa fynd skulle kunna förklara varför det är så ovanligt med infektioner i innerörat trots att det är beläget så nära det infektionsbenägna mellanörat. Det är också högst troligt att ett lokalt immunförsvar i runda fönstret påverkar transporten av läkemedel från mellanöra

till inneröra varför detta måste beaktas vid utvecklingen av lokala behandlingsmetoder med injektion till mellanörat.

I den andra studien i avhandlingen (artikel II) undersöktes tre olika geler som skulle kunna användas som bärarmaterial vid läkemedelsadministration till mellanörat. Resultaten talar till fördel för gelen hyaluronsyra (HYA-gel) som till skillnad mot de två andra gelerna carboxymetyl cellulosa och poloxamer 407 inte orsakade någon signifikant hörselnedsättning efter injektion till mellanörat på marsvin. En undersökning av hur länge gelerna stannade kvar i mellanörat liksom mikroskopisk undersökning av deras påverkan på mellan- och inneröra stödjer uppfattningen att HYA-gelen är den mest effektiva som bärarmaterial för injektion till mellanörat.

En viktig faktor för lokal behandling av innerörat genom injektion av läkemedel till mellanörat är att bärarmaterialet för läkemedlet kommer i kontakt med runda fönstermembranet. Med hjälp av magnetkamera undersöktes hur HYA-gelen fördelade sig i mellanörat på marsvin efter mellanöreinjektion liksom hur länge den kvarstannade (artikel III). HYA-gelen fördelade sig på ett förutsägbart sätt, fyllde upp mellanörat väl och stannade kvar invid runda fönstret under mer än 24 timmar. Innan injektion till mellanörat gavs gjordes ett litet hål i trumhinnan för att släppa ut luft och därmed undvika skada på runda fönstermembranet till följd av övertryck.

Hypotesen att högre koncentrationer av ett läkemedel i innerörat kan uppnås genom injektion till mellanörat jämfört med injektion till blodet undersöktes i de fjärde och femte delarbetena (artikel IV och V). Antioxidanten tiosulfat har tidigare identifierats som en lovande substans för att skydda mot skada av det för innerörat skadliga cytostatikapreparatet cisplatin. Mycket högre koncentrationer av tiosulfat i innerörat kunde i studien uppmätas efter injektion till mellanörat av substansen i HYA-gel jämfört med efter att tiosulfatlösning injicerats i blodet. Blodnivåerna av tiosulfat efter injektion till mellanörat var låga vilket bekräftar att denna metod för läkemedelsadministration till innerörat inte medför någon risk för minskad effekt av cisplatin på den tumör

man avser att behandla.

I den sista studien (artikel V) visades att en injektion av tiosulfatinnehållande HYA-gel till mellanörat på marsvin skyddar innerörat från cisplatinorsakad skada. Tiosulfatinnehållande HYA-gel injicerades till mellanörat tre timmar innan marsvinen erhöll en injektion med cisplatin i blodet. Detta bekräftar hypotesen att den utvecklade injektionsmetoden med HYA-gel som bärarsubstans för läkemedel kan användas för att behandla vissa typer av inneröronsjukdom och skada. De experimentella resultaten talar för att metoden skulle kunna användas på patienter.

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